Institut für Veterinärbiochemie und Molekularbiologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. U. Hübscher

Identification of NF-κB dependent genes which are regulated by specific coactivators

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Nadja Iveta Meier

Tierärztin
von Zürich, ZH und Buchberg, SH

genehmigt auf Antrag von

Prof. Dr. Dr. M.O. Hottiger, Referent

Prof. Dr. F. R. Althaus, Korreferent

Zürich 2006

Gewidmet

meinen Eltern, Rudolf und Iveta Meier-Osuský

Table of contents

1. Summary

Nuclear factor kappa B (NF-κB) had been suggested to play important roles in inflammatory and neurodegenerative disorders as well as carcinogenesis. NF-κB encompasses a family of inducible and widely expressed transcription factors which played a crucial role in the regulation of genes involved in immune and inflammatory responses. Over 500 target genes depending on NF-κB had been identified so far.

PARP-1, an abundant nuclear chromatin associated protein belongs to a large family of enzymes that catalyze the transfer of ADP-ribose units from its substrate β-nicotinamide adenine dinucleotide covalently to itself and other chromatin associated proteins. PARP-1 deficient mice were protected against experimentally induced septic shock, diabetes type I and Parkinsonism. This phenotype indicated that PARP-1 is important for the pathogenesis of these events. Indeed, PARP-1 was shown to be an important coactivator of NF-κB. Interestingly, recent studies had shown that the cleavage of PARP-1 by caspases could be important for the activity of PARP-1 in NF-κB-dependent disease processes.

In addition to PARP-1, many other coactivators such as the coactivator-associated arginine methyltransferase-1 (CARM-1) are also required for NF-κB-dependent gene expression.

Aim of this thesis was to investigate NF-κB-dependent gene expression in regard to their PARP-1 or CARM-1 dependency and furthermore to the caspase-mediated cleavage of PARP-1. For this purpose, gene expression levels of wild-type mouse lung fibroblasts, mouse embryonic fibroblasts and Raw264 cells were compared with the corresponding knock-out or knock-in cells upon stimulation. From over 500 target genes of NF-κB, 16 selected genes were analyzed. The results indicated that only a subset of the target genes were activated by the certain investigated coactivators. Moreover, PARP inhibitors did not influence NF-κB-dependent gene expression under the tested conditions. On the other hand, a small subset of NF-κB-dependent genes seemed to be regulated by caspase activities, potentially through the cleavage of PARP-1.

Together these results provide strong evidence for a crucial role of PARP-1 and CARM-1 in NF-κB gene expression.

1. Zusammenfassung

Der Nukleare Faktor kappa B (NF-κB) spielt eine zentrale Rolle in vielen Entzündungs- und Nerven-zerstörenden Krankheiten, aber auch in der Entstehung von Krebs. NF-κB gehört zu einer Familie von induzierbaren Transkriptionsfaktoren, welche hauptverantwortlich sind für die Regulation von Genen, die in der Immunabwehr und in Entzündungsprozessen eine wichtige Rolle spielen. Mehr als 500 verschiedene NF-κB abhängige Gene konnten bis jetzt identifiziert werden.

PARP-1 ist ein Chromatin-assoziiertes Protein, welches in hohen Mengen im Zellkern vorkommt und zu einer grossen Familie von Enzymen gehört, welche ADP-Ribose Einheiten von NAD als Substrat auf sich selber oder andere Chromatin-assoziierte Proteine übertragen. PARP-1 Knockout-Mäuse zeigen eine ausgesprochen hohe Resistenz gegenüber experimentell induziertem Septischem Schock, Diabetes Typ 1 und Parkinson Krankheit. Dieser Phänotyp ist ein klares Indiz dafür, dass PARP-1 in die Pathogenese dieser Prozesse involviert ist. In der Tat fungiert PARP-1 als wichtiger Koaktivator von NF-κB. Darüber hinaus wurde kürzlich in einer Studie gezeigt, dass die proteolytische Spaltung von PARP-1 durch Caspasen möglicherweise wichtig ist für die PARP-1 Aktivität in NF-κB abhängigen Krankheitsprozessen.

Neben PARP-1 sind einige andere Koaktivatoren wie zum Beispiel die Koaktivator assoziierte Arginin Methyltransferase-1 (CARM-1) ebenfalls essentiell für die NF-κB abhängige transkriptionelle Aktivierung von Genen.

Ziel dieser Studie war NF-κB abhängige Gene in Abhängigkeit von PARP-1 und CARM-1 zu identifizieren und darüber hinaus solche, die von der proteolytischen Spaltung von PARP-1 durch Caspasen abhängig sind. Zu diesem Zweck wurden die Expressionsstärken von Wildtyp Zellen (embryonale Mausfibroblasten, respektive Lungenfibroblasten und Raw264) mit den entsprechenden Knock-out oder Knock-in Zellen nach Stimulation verglichen. Es wurden 16 Gene von den über 500 von NF-κB abhängigen Genen untersucht. Die Resultate zeigten, dass immer nur ein Teil von den NF-κB abhängigen Genen durch die untersuchten Koaktivatoren aktiviert wird. Weiter konnte gezeigt werden, dass die Expression von NF-κB abhängigen Genen durch PARP Hemmer unter den getesteten Konditionen nicht beeinflusst wird. Andererseits scheinen einige Gene durch Caspase Aktivität, z.B. Spaltung von PARP-1, reguliert zu werden.

All diese Resultate sprechen für einen starken Beweis, dass PARP-1 und CARM-1 eine essentielle Rolle in NF-κB abhängiger transkriptioneller Aktivierung von Genen spielt.

2. Abbreviations

-/-	knock-out
+/+	wild-type
ADP	adenosine diphosphate
AGE	advanced glycated end product
ART	(ADP-ribosyl) transferase
ATP	adenosine triphosphate
Bcl-3	B-cell lymphoma protein/gene 3
BER	base excision repair
BRCT	BRCA1 carboxyl-terminal
cAMP	circular adenosine monophosphate
CARM-1	Coactivator-associated arginine methyltransferase
CBP	CREB binding protein
CREB	cAMP response element binding protein
cDNA	circular deoxyribonucleic acid
CIA	Collagen-induced arthritis
CIA genes	constitutively and immediately accessible genes
CNS	central nervous system
COX	Cyclooxygenase
DBD	DNA binding domain
DEPC	diethyl pyrocarbonate
$DEVD_{214}$	Caspase 3, and 7 recognition/cleavage motif
DNA	deoxyribonucleic acid
EBV	Epstein-Barr Virus
eNOS	Endothelial nitric oxide synthetase
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
Glut-2	Glucose transporter 2
GM-CSF	granulocyte macrophage colony stimulating factor
GRIP-1	glucocorticoid receptor-interacting protein-1
GRR	glycine-rich region
HIV	human immunodeficiency virus
HPRT	hypoxanthine ribosyltransferase
HTH	helix-turn-helix domain
ICAM-1	intercellular adhesion molecule-1
IFN	Interferon
IgG	immunoglobulin G
IKK	IκB kinase
iNOS	inducible nitrite oxide synthetase
IP-10	interferon-inducible protein-10 or immune protein-10
IRAK	IL-1 receptor-associated kinase
IκB	Inhibitor of nuclear factor kappa B
KC	keratinocyte derived chemokine
kDa	kilo Dalton
KI/KI	knock-in
LPS	Lipopolysaccharide
LZ	leucine zipper
MAPKK	Mitogen activated kinase kinase
MCP-1	monocyte chemoattractant protein-1

MEF	mouse embryonic fibroblast
Mg	Magnesium
$MgCl_2$	Magnesium chloride
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLF	mouse lung fibroblast
MOF	Multiple organ failure
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
NAD	nicotinamide adenine dinucleotide
NEO gene	neomycin resistance gene
NIK	NF-κB-inducing kinase
NK-cell	Natural killer cell
NLS	nuclear localization signal
nNOS	neuronal nitric oxide synthetase
NO	nitrite oxide
NSAID	non-steroidal anti-inflammatory drug
PAF	platelet activating factor
PARP	poly(ADP-ribose) polymerase
PCR	polymerase chain reaction
PDGF	Platelet derived growth factor
PF	platelet factor
PGN	Peptidoglycan
PJ-34	N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide
PKA	protein kinase A
PMA	phorbol myristate acid
PMN	polymorphonuclear neutrophil
RANTES	regulated upon activation, normal T-cell expressed, and presumably secreted
Rel	reticuloendotheliosis linked
RHD	Rel homology domain
RIP	receptor-interacting protein
RT	reverse transcription
RT-PCR	reverse transcription-PCR
Ser	Serine
SZT	Streptozotocin
TAK	TGF-associated kinase
TANK	Tankyrase
T-BP	tumor necrosis factor binding protein
TGF	transforming growth factor
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRADD	TNF receptor-associated death domain
TRAF	TNE receptor-associated factor
TRF1	telomer regulatory factor
ZF	zinc finger

3. Introduction

3.1. Transcriptional factor nuclear factor kappa B (NF-κB)

Nuclear factor kappa B (NF-κB) is a widely expressed, inducible transcription factor of particular importance to cells of the immune system [reviewed in ref. 1]. The active NF-κB transcription factor promoted the expression of over 500 target genes [2]. It played a crucial role in the regulation of gene expression of many genes involved in mammalian immune and inflammatory responses, including cytokines, cell adhesion molecules, complement factors, and a variety of immune receptors [reviewed in ref. 3]. NF-κB also mediated the transcriptional activation of anti-apoptotic genes and was therefore essential for the survival of cells under certain conditions [4]. Moreover, it was active in tumor cells and required for rapid growth of these cells [5]. In addition, some viruses were shown to hijack the NF-κB signaling/transcriptional system for the activation of their own genes [6] or even influence the expression of host genes by manipulation of the NF-κB activity [7-9].

Abnormalities in the regulation of NF-κB activity were tightly linked to the pathogenesis of inflammatory disorders including septic shock, radiation damage, myocardial infarction, acute-phase reactions, and diabetes, as well as chronic inflammatory diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease [4].

3.1.1. Family of NF-κB/Rel- and IκB-proteins

3.1.1.1. NF-κB/Rel-proteins

NF-κB is expressed in most cell types. The Rel family members include p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), which form homo- and heterodimers (see Figure 1).

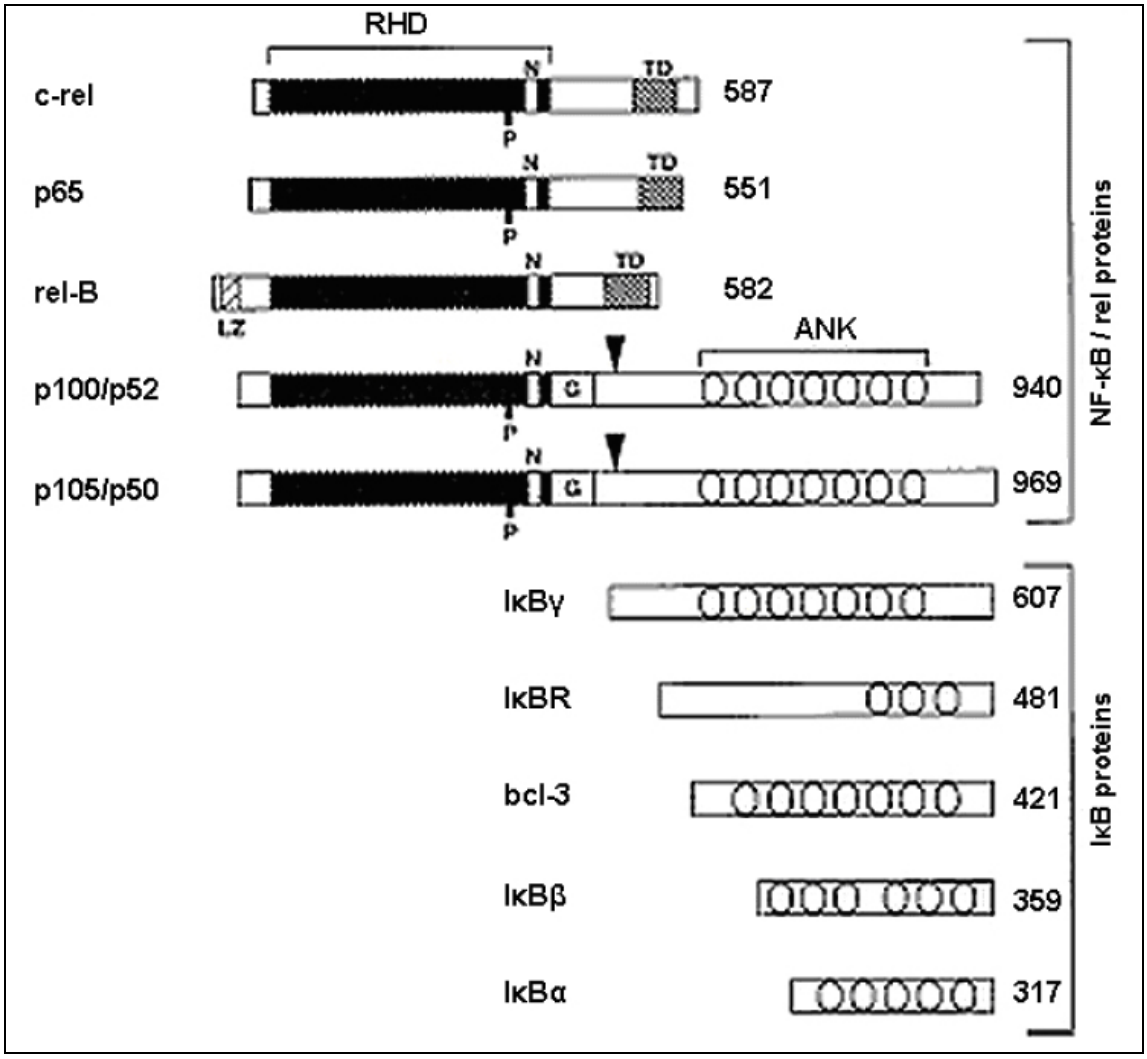


Figure 1. Members of the mammalian Rel/NF-κB and IκB protein family: The number of amino acids in each protein was shown on the right. The arrows point to the endoproteolytic cleavage sites of p52/p100 and p50/p105. RHD, Rel homology domain; N, nuclear localization signal; P, PKA phosphorylation motif; TD, transactivation domain; LZ, leucine-zipper domain of Rel B; G, glycine-rich region; ANK, ankyrin repeats [modified 1].

The prototypical and most studied form, NF-κB, is the 'classical' heterodimer consisting of the two subunits p50 (NF-κB1) and p65 (RelA) [10]. When these two subunits are coexpressed at comparable levels in the cell, they are preferentially forming the 'classical' NF-κB p65/p50 heterodimer, generally considered to be the predominant, inducible form of NF-κB in most cells [reviewed in ref. 1]. This heterodimer had a high affinity for the consensus NF-κB DNA sequence 5'-GGGRNNYYCC-3' (where R was purine and Y was pyrimidine).

All proteins of the Rel family shares a conserved 300-amino acid region within their amino termini, termed the Rel homology domain (RHD) [11]. The

RHD is responsible for dimerization, DNA-binding, translocation to the nucleus and interactions with heterologous transcription factors.

The active DNA-binding form of NF-κB is a dimer. Almost all combinations of Rel/NF-κB homo- or heterodimers had been identified in many different cell types [1]. One exception is RelB, which only forms heterodimers with p50 or p52 [12, 13]. Not all dimers of Rel/NF-κB proteins are transcriptionally active, and the members of this family are divided into two groups based on their transactivation potential. The first group, including RelA, c-Rel and RelB contains a strong transactivation domain within the C-terminal sequence [12, 14-18]. In addition to its C-terminal transactivation domain, RelB also contains an N-terminal leucine zipper-like transactivation domain. Both N- and C-terminal domains of RelB were required for full transcriptional activity [19]. In contrast, the second group composing of p50 and p52, does not contain any transactivation domains, and thus, generally does not act as transcriptional activators. Indeed, homo- or heterodimers of p50 and p52 could repress NF-κB-dependent transcription [15, 20-23]. The mechanism by which p50 or p52 homodimers inhibit transcription is not completely understood. One interesting property of p50 and p52 homodimers is their ability to specifically associate with the protooncoprotein Bcl-3, a member of the IκB family, through the interactions between the RHD of p50 or p52 and the ankyrin repeats of Bcl-3 [24-29]. This protor complex was shown to either activate or repress NF-κB-dependent transcription.

In contrast to the other Rel/NF-κB members, p50 and p52 are synthesized as precursor molecules of 105kDa (p105) and 100kDa (p100), respectively [30]. The N-terminal regions of p105 and p100 constitute the RHD of p50 and p52. Directly adjacent to the RHD is a glycine-rich region (GRR) followed by the IκB homologous C terminus [31]. The C-terminal sequences of p105 and p100 contain multiple copies of the ankyrin repeat motifs, which were also described in all IκB family members. Indeed, p105 and p100 could both inhibit upon dimerization the nuclear localization and transcriptional activity of Rel/NF-κB proteins. Generation of p50 and p52 from p105 and p100 occurs by a proteolytical processing mechanism [32].

3.1.1.2. Structure of NF-κB

The geometry of NF-κB dimers, when bound to DNA, resembles that of a butterfly with the cylinder of DNA trapped within its wings (see Figure 2) [1].

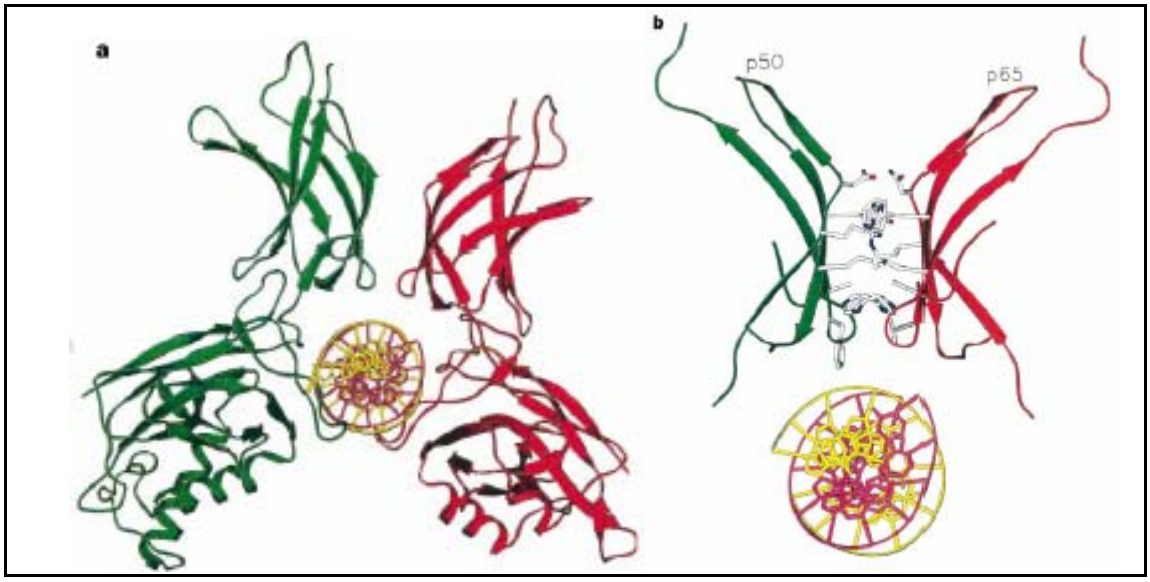


Figure 2. The structure of the heterodimer bound on DNA. a, Ribbon drawing of the entire complex, viewed down the DNA helical axis. The p50 subunit is in green and the p65 subunit is in red. The top strand of DNA is in pind, and the bottom strand is in yellow. b, The hydrophobic core of the dimer interface between p50 (green) and p65 (red) consists of an array of nonpolar hydrocarbons, aromatic rings and uncharged polar residues pointing from the β-sheets in towards the interface [33].

The RHD consists of two domains of anti-parallel β-sheets packed into a sandwich structure that resembles the structure of the immunoglobulin [34]. Dimerization of two Rel/NF-κB molecules is mediated by an extensive hydrophobic surface formed between two β-sheets of the C-terminal globular domain in the RHD [35, 36]. The interaction of such a complex with DNA involves 10 loops of α-strands at the N-terminal domain of the RHD. This extensive contact with DNA explains the high affinity for DNA observed for the different NF-κB members. Although loops from both the N- and C-terminal sequences of the RHD contact DNA, the majority of the contacts are confined to the N-terminal sequences. The advantage of using loops to contact DNA might lies in the inherent flexibility of the loops, which could allow NF-κB to interact with a greater variety of DNA sequences [30]. A further component of the RHD of all Rel/NF-κB proteins is a short stretch of basic amino acids that forms the nuclear localization signal. The NLS is located close to the C-terminal end of the RHD and mutational analysis confirmed the importance of

this region for the transport of RelI/NF-κB complexes from the cytoplasm to the nucleus [1].

3.1.2. IκB-proteins

In unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor complex by its physical association with one of the several inhibitors of NF-κB (IκBs). These proteins also comprise a structurally and functionally related family of molecules, which include IκBα (37kDa), IκBβ (43kDa), IκBγ, IκBε, p105/p50 (C terminus), p100/p52 (C terminus), IκB-R, and Bcl-3 [reviewed in refs. 37, 38].

All known IκB proteins contain multiple copies of a 30-33 amino acid sequence originally discovered in the SW16 protein of *Saccharomyces cerevisiae* and named SW16/ankyrin repeats. As described above, the p50 and p52 precursor molecules p105 and p100 also contain ankyrin repeats in their C-terminal regions and are capable of inhibiting NF-κB activity [31]. All members of the IκB family express between three and seven ankyrin repeats.

A number of studies reveled that separate IκB molecules preferentially inhibited distinct RelI/NF-κB protein dimers. For example, IκBα and IκBβ preferentially interacted with dimers containing p65 and had been shown to be the main functional modulators of the ‘classical’ NF-κB p65/p50 heterodimer [reviewed in refs. 37, 38]. Both IκBα and IκBβ did not only sequester NF-κB in the cytoplasm, but also inhibited the DNA binding activity of NF-κB [39]. Interestingly, the only IκB molecules, which could associate with RelB are p100, p105, and the 46kDa C-terminal portion of p100 (tentatively named IκBε), which all could effectively inhibit p52/RelB heterodimers [40]. The subunit composition of a given NF-κB complex therefore influences its subcellular localization, transactivation potential and mode of regulation. For example, the prototypical NF-κB complex, composed of p50/p65 heterodimers, was primarily cytoplasmic. In contrary, homodimers of p50/NF-κB1 were preferentially shown to be nuclear proteins [41].

Inhibition of RelI/NF-κB proteins by IκB molecules occurs via protein-protein interactions between the ankyrin repeats of IκB and regions of the RHD. With the exception of Bcl-3 and an unphosphorylated form of IκBβ, this

interaction enables the IκB proteins to mask the nuclear localization signal (NLS) and prevents nuclear translocation of RelI/NF-κB dimers. Although ankyrin repeats are crucial for IκB to bind to RelI complexes, not all ankyrin repeats are required, since mutation of the third ankyrin repeat of IκBα had no effect on its ability to inhibit NF-κB. In addition to the centrally located ankyrin repeats of IκB proteins, the N- and C-terminal domains of each IκB exhibit important structural and functional characteristics. Within their C-termini, most IκB proteins contain an acidic and Thr-rich sequence, which was thought to play a role in stabilization of the molecule and in the prevention of NF-κB DNA-binding activity. The N-terminal domain contains sites of serine phosphorylation and ubiquitination, which are important for signal-induced degradation, at least for IκBα and IκBβ [1] (see also section 3.1.4). Signals that induced NF-κB activity cause the dissociation and subsequent degradation of IκB proteins, allowing NF-κB dimers to enter the nucleus and induce gene expression.

3.1.3. Activators and inhibitors of NF-κB-dependent gene expression

In most cell types, nuclear NF-κB activity is induced by exposure to a wide variety of viruses, bacteria or bacterial products. It seems likely that a virus would gain a selective advantage from the acquisition of κB binding site in its promoter. Indeed, many viruses also harbor NF-κB binding sites in their viral promoters. If the transcription factor is induced either directly through viral infection or indirectly by the ensuing immune response, the κB sites-containing viral promoter would be transactivated, resulting in enhanced viral transcription [3]. Gram-positive and gram-negative bacteria and their cell wall components activate the innate immune system of the host and induce secretion of proinflammatory molecules, mainly chemokines and cytokines. Chemokines are the main proinflammatory mediators induced in monocytes by bacteria through peptidoglycan (PGN) and lipopolysaccharide (LPS), the main cell wall components of gram-positive and gram-negative bacteria. Gram-positive bacteria, PGN and LPS activate pathways through pattern recognition receptors, which result in the activation of NF-κB [42]. The observations that distinct eukaryotic parasites (e.g. Theileria parva) could

activate NF-κB [43] are of great veterinary interest. NF-κB activity is also induced during various physiological stress conditions such as ischemia/reperfusion, liver regeneration and hemorrhagic shock [2]. Besides physiological stress situations, the human body is exposed to environmental hazards and therapeutic drugs, which could also exert a stress. Indeed, NF-κB was reported to be activated both by environmental stresses, such as heavy metals or cigarette smoke, and by therapeutic drugs, including various chemotherapeutic agents [2].

The NF-κB activity could also be negatively regulated by different cytokines, such as IL-4, IL-10, IL-11, IL-13, antioxidants, such as glutathione, vitamin E or thioredoxin, as well as anti-inflammatory drugs such as glucocorticoids, acetylsalicylic acid or cyclosporine A [44-51].

Bacteria	Mycobacterium tuberculosis, Helicobacter pylori, Lactobacilli, Listeria monocytogenes, Salmonella dublin, Salmonella typhimurium, Staphylococcus aureus
Bacterial products	Exotoxin B, Lipopolysaccharide (LPS), Staphylococcus enterotoxin A and B, Toxic Shock Syndrome Toxin I
Viruses	Epstein-Barr Virus (EBV), Hepatitis B Virus, HIV-1, Herpes Simplex Virus-1, Influenza Virus, Newcastle disease virus, Bovine papillomavirus, Bovine leukemia, Cowpox virus, Adenovirus
Eukaryotic parasite	Theileria parva
Cytokines	IL-1, IL-2, IL-12, IL-15, IL-17, IL-18, TNF-α
Physiological (stress) conditions	Adhesion, Depolarization, Hemorrhage, Hyperglycemia, Hyperosmotic Shock, Hypoxia, Ischemia (transient, focal), Liver regeneration, Reoxygenation, T-cell Selection
Physical stress	Ultraviolet irradiation (UV-A, B, C), γ Radiation
Oxidative stress	Hydrogen Peroxide, Ozone, Reoxygenation
Environmental hazards	Chromium, Cigarette Smoke, Cobalt, Nickel, Silica Particles
Therapeutic used drug	Cisplatin, Phenobarbital
Modified proteins	Advanced glycated end products (AGEs)
Overexpressed proteins	Ig heavy chain, MHC Class I
Mitogens, growth factors and hormones	Follicle stimulating hormone, Insulin, M-CSF, Serum
Physiological mediators	Angiotensin II, Bradikinin, Collagen Type I, Hemoglobin, Leukotriene B4, PAF (platelet activating factor), Thrombin
Chemical agents	Phorbol ester

Table 1. Partial list of NF-κB activators [3].

3.1.4. Signal cascade of NF-κB

The pathways of how cytokines and pathogens activate NF-κB have been extensively investigated. Cytokine receptors and pathogen pattern recognition receptors, such as Toll-like receptors (TLRs) are single-pass, transmembrane

receptors lacking intrinsic kinase activity. Upon binding of the corresponding ligands, the receptors form dimeric, trimeric or tetrameric structures, resulting in intracellular aggregation of the cytoplasmic signaling domains. This results in the recruitment and activation of a submembranous IκB-kinase (IKK) signalosom. The cytosolic multiprotein IκB-kinase complex of 700 kDa is composed of catalytic basic helix-loop-helix-containing kinases, IKKα, IKKβ and a regulatory subunit, IKKγ, required for coupling IKK to upstream activating kinases [10]. In the IKK complex, the catalytic IKKβ subunit is largely thought to be responsible for site-specific serine phosphorylation of IκBα in its N-terminal regulatory domain, representing in the first rate limiting process of NF-κB activation.

The composition of receptor and signalosome complexes varies and is dependent on the signaling pathway. For instance, in the case of IL-1, the signal adapters include MyD88, IL-1 receptor-associate kinase (IRAK), and the TNF-α receptor-associated factor 6 (TRAF6) [52]. TRAF6 was required for the coupling of activated IL-1 receptors to IKK. TRAF6 was also required to activate the downstream MAPKK kinases, TGFβ-associated kinase (TAK1) [52]. TAK1, in turn, phosphorylated the NF-κB-inducing kinase (NIK), resulting in IKKβ phosphorylation and IKK activation [52]. In the case of the TNF signaling, TNF receptor-associated death domain (TRADD), TRAF2, and receptor-interacting protein (RIP) constitute the submembranous signaling complex. The RIP kinase plays an indispensable role in IKK activation, as targeted disruption of RIP abolished NF-κB activation. RIP recruits the IKK signalosome to the TNF receptor I by binding IKKβ, thus directly recruiting the cytosolic IKK to the receptor. IKKα and IKKβ are subsequently phosphorylated, which results in the release of activated IKK complex back into the cytosolic fraction [52] (see Figure 3).

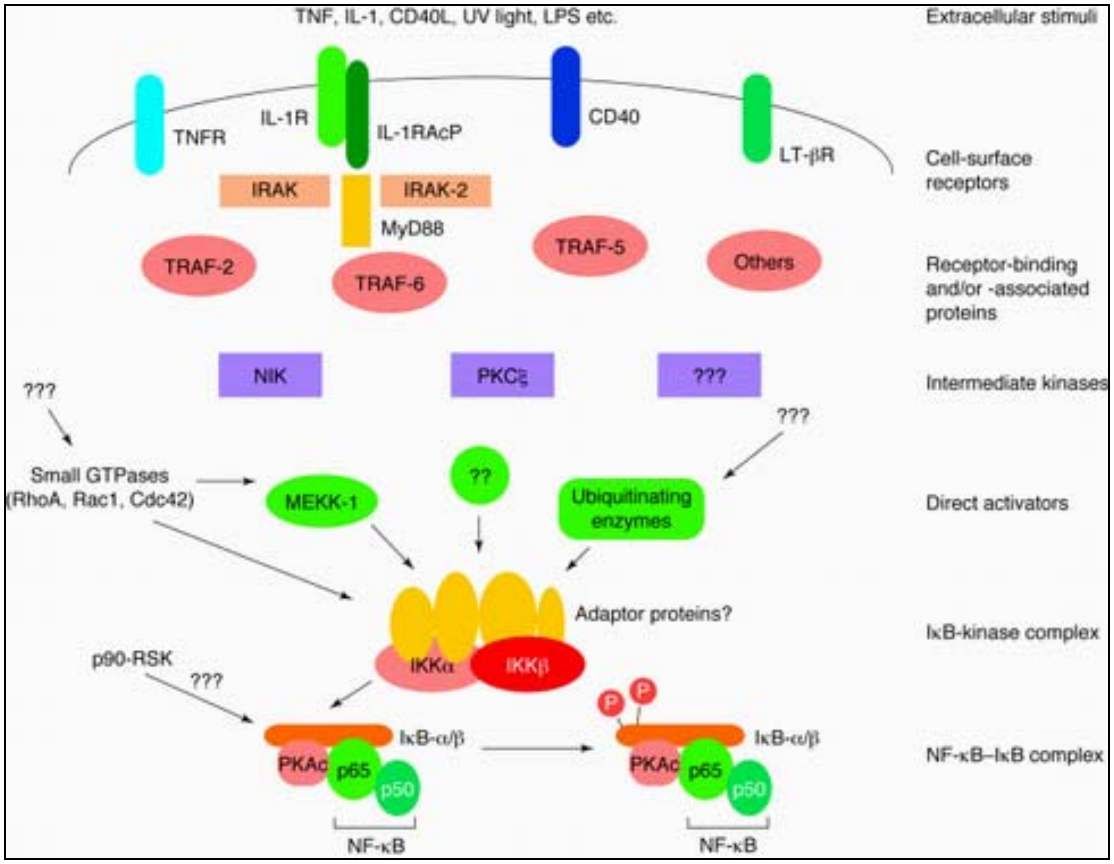


Figure 3. This diagram portrays the signal transduction pathway in simplified terms. Abbreviations: CD40L, CD40 ligand; IKKα/IKKβ, IκB-kinase α- and β-subunits; IL-1, interleukin-1, IL-1R, IL-1 type 1 receptor; IL-1βRAcP, IL-1 accessory protein; IRAK, IL-1 receptor associated kinase; LPS, lipopolysaccharide; LT-βR, lymphotoxin β receptor; MEKK-1, mitogen-activated protein kinase/extracellular signal-regulated kinase 1; NID, NF-κB-inducing kinase; p90-RSK, p90-ribosomal S6 protein kinase; p, phosphate; PKAc, protein kinase A catalytic subunit; PKCζ, protein kinase Cζ; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNFR-associated factor; UV, ultraviolet [53].

In most cases, extracellular and intracellular NF-κB activating signals converge on the multiprotein IKK complex, which phosphorylates IκBα on two serine residues (Ser in position 32 and 36). Phosphorylated-IκB dissociated from NF-κB and is specifically bound to a complex initiating IκB ubiquitination and proteolysis [10]. A very unusual pathway that induces IκB degradation through cytoplasmic calpains also has been identified during infection with certain viruses [41]. Following IκB proteolysis, liberates cytoplasmic NF-κB to rapidly enter the nucleus and to bind to specific response elements in the regulatory regions of its target promoters/enhancers.

Promoter/enhancers-bound NF-κB activates transcription through the assembly of larger nucleoprotein complexes termed enhanceosomes. Transcriptional activation is the primary mechanism controlling gene

expression in response to tissue specific-, developmental- and hormonal-induced cellular signaling [52]. Inducible transcriptional regulation is a multistep process involving the cooperative assembly of architectural, sequence-specific transcription factors, and coactivator/bridging factors on a target promoter/enhancer. Formation of the promoter assembly complexes /enhanceosomes also regulates in the recruitment and cooperative binding of chromatin-remodeling proteins [52]. The presence of chromatin-remodeling factors activates the preinitiation complex that, in turn, controls RNA polymerase II activity and subsequent gene expression. The phenomenon of binding cooperativity allowed highly inducible genes to be expressed in the setting of limiting concentrations of transcription factors [52].

Although the basic biochemistry of preinitiation complex formation and the mechanisms of how signaling molecules control transcription factors had been extensively investigated, the spectrum of genes controlled by specific signaling pathways within the context of complex biological stimuli remains to be explored systematically [52].

3.1.5. Coactivators of NF-κB

Activator dependent upregulation of class II gene transcription *in vivo*, required a multiply of activities and physical interactions between chromatin remodeling factors, RNA-polymerase II (Pol II) and its associated general transcription factors (GTFs; TFIIA, B, D, E, F and H), mediator complexes, TATA binding protein (TBP) associated factors (TAFs), and other less characterized cofactor/coactivator complexes including the USA derived positive cofactor [reviewed in ref. 4].

Enhanceosome formation involves coactivator recruitment, proteins required for the control of core promoter activity at a distance. Coactivators do not bind DNA themselves but allowed transcription factors to interact with the basal transcriptional apparatus and induce chromatin remodeling [4, 54-56].

A growing number of coactivators were described to be required for full NF-κB-dependent gene expression. The specific sets/combinations of coactivators used at a given gene promoter varies and is dependent on the cell type and stimulus. It was recently shown that poly(ADP-ribose)

polymerase 1 (PARP-1) acts as a coactivator of NF-κB in concert with the classical NF-κB coactivator p300/CBP [56]. PARP-1 could directly bind to both subunits of NF-κB, p50 and p65, by protein-protein interaction.

Once in the nucleus NF-κB will activate genes dependent on the availability of coactivator sets in the cell. It is conceivable that not only the simultaneous activation of other transcription factors such as AP-1, ETS, C/EBPβ, STAT-1 and p53 might influence the spectrum of induced NF-κB-dependent genes but also the availability of different coactivators and cofactors of NF-κB, thereby determining whether activation of NF-κB leads to cell survival, necrotic cell death or apoptosis [56]. Previous reports have shown that NF-κB-dependent transcriptional complexes do not only require the presence of the transcriptional coactivator p300 or its structural homologue, CBP, but also the p300/CBP-associated factor (P/CAF) and the p160 family of steroid receptor coactivators [4].

It was thought that the critical role of p300/CBP and its associated coactivators is to promote the rapid formation of the pre-initiation and re-initiation complexes which facilitate multiple rounds of transcription by modifying the amino-terminal tails of nucleosomal histones and bridging NF-κB to the general transcriptional machinery. Since other sequence specific transcription factors also bind to p300/CBP and their associated coactivators and the expression levels and localization of these coactivators varies between different cell types, these coactivators are generally limiting *in vivo*. Additional components might be required to stabilize the association of distinct NF-κB coactivator complexes. Although the recruitment of p300 or CBP to NF-κB-dependent enhanceosomes was required for synergistic activation, tethering p300/CBP alone to the promoter through NF-κB might not to be sufficient for full activity of NF-κB in the context of chromatin. Several reports indicated that the combined actions and interactions of distinct transcriptional coactivator complexes and cofactors seemed to be attributable to the strong transcriptional activity of NF-κB, depending on the stimuli and the cell type [reviewed in ref. 4].

3.2. PARP-1

PARP-1 plays an essential and central function in the activation of NF-κB-dependent target genes during the inflammation processes in classical inflammatory diseases as well as cardiovascular and neurodegenerative disorders. Remarkably, the fact that PARP knockout (-/-) mice did not show any developmental defects, especially regarding the immune system, indicated that a multitude of NF-κB-dependent genes could be activated also in the absence of PARP-1 under normal physiological conditions [57].

Mammalian PARP-1, an abundant 114-kDa nuclear chromatin-associated protein, belongs to a large family of enzymes that catalyzes the transfer of ADP-ribose units from β-nicotinamide adenine dinucleotide (NAD^+) onto glutamic acid residues of nuclear protein acceptors [4].

Activation of PARP-1 was proposed to be one of the earliest responses of mammalian cells to genotoxic stress [reviewed in refs. 58, 59]. The enzymatic activity of PARP-1 was strongly stimulated in the presence of nicks and double strand breaks in DNA [reviewed in ref. 60]. These observation had contributed to the idea that PARP-1 might act as a 'molecular nick sensor', thereby mediating stress-induced signaling in the presence of DNA lesions in an NAD^+-dependent manner to downstream effectors involved in coordinating the cellular response to DNA damage [reviewed in refs. 60-63]. PARP-1 recognizes and rapidly binds to DNA strand breaks through its zinc fingers. The catalytic domain of PARP-1 is allosterically activated and starts to synthesize complex branched poly(ADP-ribose) chains, resulting in automodifications of PARP-1 and probably extensive modification of histones at sites of DNA strand breaks. Modification of chromatin proteins and PARP-1 itself might subsequently function as a strong signal that might rapidly recruit other DNA damage-signaling molecules [63]. Reported modified proteins included topoisomerase I and II, histones, p53, and high-mobility group proteins [64-66 and reviewed in ref. 67]. In intact organisms, PARP-1 itself is the predominant acceptor of poly(ADP-ribose) [reviewed in ref. 60]. Only a few of the proposed substrates of PARP-1, such as p53, topoisomerase I, and histone I, have been shown to directly interact with PARP-1 [68-70].

It was suggested that PARP-1 plays a crucial role in DNA replication, DNA base excision repair (BER), V(D)J recombination and as regulation of telomere length [reviewed in refs. 60, 71]. Other functions proposed for PARP-1 include gene expression, chromatin organization, proliferation and differentiation, cellular NAD^+ metabolism, and apoptosis [reviewed in refs. 71, 72]. Additionally, PARP-1 was thought to serve also as a marker for apoptosis [73, 74]. However, the physiological function of PARP-1 is still under heavy debate.

3.2.1. The PARP family

In addition to the 'classical' 113-kDa PARP-1, more than 16 novel PARP like genes existed in mammalians. These new PARPs were structurally distinct from the PARP-1 and could be classified together with PARP-1 according to their structures and sizes into at least 7 subgroups [4,75,76] (see Figure 4). The novel PARP family members seemed to be involved in specific functions requiring limited levels of poly(ADP-ribosyl)ation [77]. PARP members of class II and III beared the strongest resemblance to PARP-1 [75, 76, 78, 79]. PARP-2 seems to play an important role in the response to DNA damage [78]. Interestingly, in contrast to PARP-1 and PARP-2, the PARP activities of these new members seemed not to be dependent on DNA strand breaks [80].

Tankyrase-1 and 2/3 (TANK-1 and -2), two mammalian proteins with PARP activity, were identified as components of the telomere complex [81-83]. These two proteins of class III were proposed to regulate the length of telomeres by modulating the activity of TRF1, a negative regulator of telomere length [81-83]. Over-expression of TANK-2/3, in contrast to TANK-1, caused rapid necrotic cell death in the absence of DNA damage [82].

The functions of the other PARP family members of classes IV-VII are not yet known.

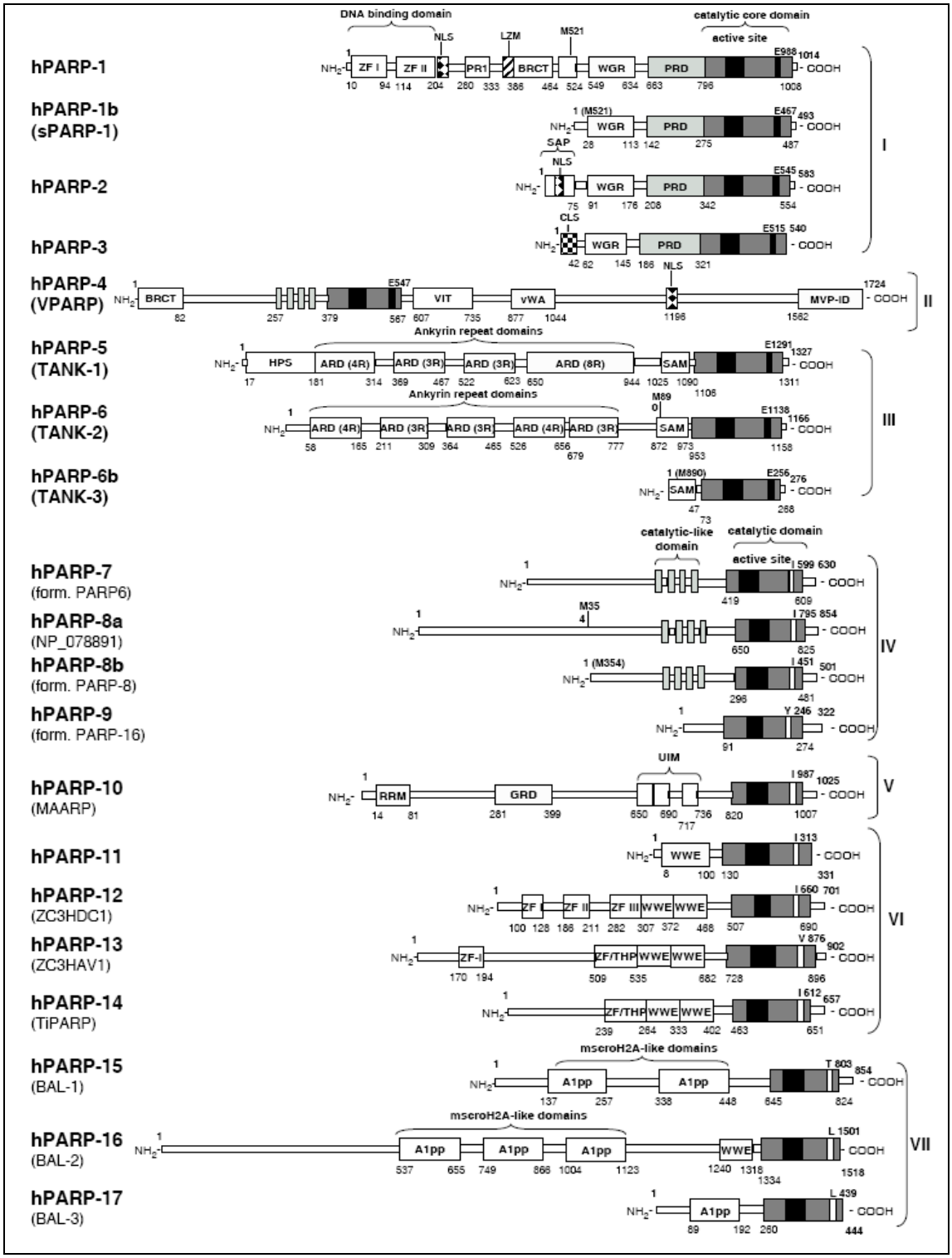


Figure 4. The structures of the PARP family members [76].

3.2.2. Structure of PARP-1

PARP-1 is a highly conserved enzyme found in all multicellular lower and higher eukaryotes [60] and consists of three domains [reviewed in refs. 60, 84]:

- a DNA binding domain (DBD) containing a bipartite nuclear localization signal (NLS) which is interrupted by a caspase cleavage site
- an automodification domain
- a catalytic domain which is the most highly conserved region of the PARP molecule

The DNA binding domain contains two zinc fingers (FI and FII) and two helix-turn-helix (HTH) motifs [85, 86] (see Figure 5).

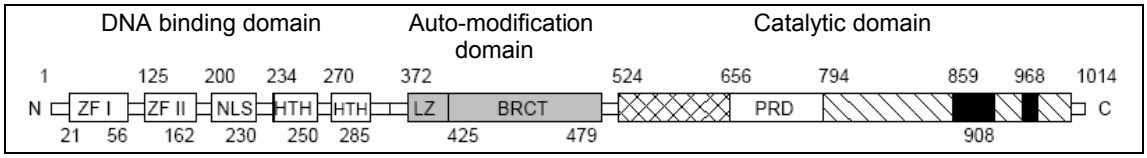


Figure 5. The structure of PARP-1; Abbreviations: ZF, zinc finger; NLS, nuclear localization signal; HTH, helix-turn-helix domain; LZ, leucine zipper; BRCT, BRCA-C-terminal domain; PRD, PARP regulatory domain [4].

These two zinc fingers are the main structures responsible for binding to double-strand breaks or single-strand breaks and for the activation of PARP-1 enzyme activity [85, 86]. A moderate non-specific association of PARP-1 with non-damaged DNA had been proposed to depend most probably on the HTH motifs [87]. Moreover, the zinc fingers could also act as an interface with various protein partners [reviewed in ref. 71].

The automodification domain contains a leucine zipper (LZ) motif in the N-terminal part and a BRCA1 carboxyl-terminal (BRCT) protein interaction domain in the C-terminal part [88]. The LZ and BRCT domains are involved in protein-protein interactions [89, 90]. The LZs might be responsible for homodimerization of PARP-1 [88, 91]. The automodification domain contains possible auto-poly(ADP-ribosyl)ation sites implicated in the negative regulation of interactions between PARP-1 and DNA [92-94].

The catalytic domain of PARP-1 was located in the C-terminal part of the enzyme. The N-terminal domain of the catalytic domain, which comprises the highly conserved PARP regulatory domain, consists five α-helices and one

3_{10}-helix [95]. The C-terminal domain of the catalytic domain shares several structural features with mono(ADP-ribosyl) transferases including an evolutionarily conserved region, called the 'PARP signature' [96].

PARP-1 was thought to be a special member of the (ADP-ribosyl) transferase (ARTase) superfamily since it was suggested to mono(ADP-ribosyl)ate a protein, comparable to other ARTases, and subsequently to elongate this modification to polymers.

The 15-kDa region between the automodification and the minimal catalytic domain has not yet been extensively characterized and its function is still unknown [61].

3.2.3. Localization of PARP-1

The PARP-1 gene is constitutively expressed in testis, spleen, brain, thymus, intestine, colon, and nasal cavities [97, 98]. Very high levels of PARP-1 were found in lymphoid organs, especially thymus, in the germinal centers of the spleen, and in the Peyer's patches in the ileum, while only very low levels of PARP-1 expression were found in organs such as liver, kidney, and heart [97-99]. In the central nervous system (CNS), PARP-1 was highly expressed in regions with a high neuronal cell density such as hypocampal neurons, granule cells of the dentate gyrus, Purkinje cells of the cerebrellar cortex, as well as microglia and astrocytes in several regions [99, 100]. For non-neuronal cell types, a direct correlation could be observed between cell proliferation and high expression levels of PARP-1.

An increase in PARP-1 mRNA levels was observed during thymocyte proliferation and upon activation of lymphocytes and peripheral blood mononuclear cells [101, 102]. The tissue-, cell- and cell cycle-specific expression pattern of PARP-1 strongly suggests that PARP-1 is not only critical to major cellular functions but that its expression is also modulated through complex transcriptional regulation [4].

PARP-1 exclusively localizes to the nucleus, where it is not homogeneously distributed [103]. The exact localization in the nucleus is still under debate. It was shown that PARP-1 associates with nuclear matrix regions and localizes to centromers during metaphase [104], while other

studies indicated that PARP-1 is preferentially found in nucleoli and defined nuclear bodies [103, 105].

Treatment of cells with RNA synthesis inhibitors caused PARP-1 to become evenly distributed throughout the nucleus [105]. The association of PARP-1 with actively transcribed regions in the chromatin strongly implies a role for PARP-1 in transcription [4]. Surprisingly, treatment with DNA synthesis inhibitors did not change the distribution of PARP-1 in the nucleus [105].

3.2.4. Enzymatic activity of PARP-1

Genetic approaches clearly showed that poly(ADP-ribosyl)ation was neither affecting the DNA binding activity of NF-κB nor required for NF-κB-dependent gene expression [4, 56, 106,107]. This seemed on the first hand not to be compatible with reports describing an inhibitory effect (15 to 40%) of inhibitors of poly(ADP-ribose)polymerases and mono(ADP-ribosyl) transferases on the expression of inflammatory mediators in mice [108-114, and reviewed in refs. 4, 115]. However these observations might be explained in three ways: First, it was known that 3-AB and nicotinamide as well as novel types of PARP-inhibitors not only inhibit the enzyme activity of PARP-1 but also of other poly(ADP-ribose) polymerases such as PARP-2 or TANK-2 [4, 82], or even mono(ADP-ribosyl) transferases [116-118], which were also described to play a crucial role in inflammatory response pathways [116-118]. Indeed, poly(ADP-ribose) formation was drastically reduced only in PARP-1 -/- brain, pancreas, liver, small intestine, colon, and testis, whereas still moderate levels of residual poly(ADP-ribose) formation could be observed in PARP-1 -/- stomach, bladder, thymus, heart, lung, kidney and spleen [4, 115, 119]. Secondly, based on recent reports, one could not exclude the possibility that the novel types of PARP-inhibitors might even inhibit non PARP-like targets such as Akt/PKB or MMP's [4, 120]. Third, the enzymatic activity of PARP-1 might be required for transcriptional activity of other transcription factors involved in these inflammatory processes. Several groups had shown that co-operative activities between transcription factors such as AP-1, STAT-1 or IRF-1 in the enhanceosomes of NF-κB-dependent genes including iNOS,

ICAM, COX and IFNβ and IFNγ, were required for full synergistic activation of these genes [121-124]. Indeed, in human endothelial cells, the PARP inhibitor 3-AB reduced oxidant-induced binding activity of the transcription factor activator protein 1 (AP-1) to the promoter of ICAM-1 [125]. Finally, a very recent report indicated that PARP inhibitors, DHIQ and 3-AB, possess free radical scavenging properties [126]. Since inflammation or stroke were related to oxidative stress it is quite possible that PARP inhibitors with antioxidative potency contribute indirectly and non-specifically to decreased NF-κB-dependent transcriptional activity by reducing free radicals. Together, it is rather unlikely that poly(ADP-ribosyl)ation is directly required for repression or stimulation of the transcriptional activity of NF-κB under physiological conditions *in vivo*.

Although the proximal events and cellular inflammatory networks in several inflammatory diseases were quite different, they all had some common features characterized by the activation of NF-κB, the production of pro-inflammatory mediators and oxygen free radicals as well as subsequent culmination in activation of the PARP-1-"suicide-pathway" [reviewed in refs. 4, 71, 106, 127-131]. Thus, PARP-1 might function in these pathophysiological processes at two different levels, firstly through its coactivator function for NF-κB and secondly by depleting the intracellular NAD^+ and ATP levels which result in necrotic cell death and tissue damage. Pharmacological inhibition of PARP-1 improved the adverse clinical effects in different pathologies associated with inflammation after cell death [108-114, 134]. Since the enzymatic and DNA binding activity was not required for NF-κB-dependent transcriptional activation after treatment of cells with pro-inflammatory or genotoxic substances, our lab proposed that the observed anti-inflammatory effects of the PARP inhibitors did not influence PARP-1 coactivator function but only inhibited the NAD^+ and ATP depletion and subsequently also necrotic cell death and tissue damage.

3.2.5. PARP cleavage by caspase

A very recent study presented evidence that PARP-1 was cleaved during inflammation by unknown caspase-like proteases [135]. For this study, a PARP-1 knock-in (PARP-1 KI/KI) mice model was generated, expressing a PARP-1-DEVD(214) mutant protein, which was resistant to cleavage by caspases. While PARP-1 KI/KI mice developed normally, they were highly resistant to endotoxic shock and to intestinal and renal ischemia-reperfusions, which were associated with reduced inflammatory responses in the target tissues and cells due to the compromised production of specific inflammatory mediators [135]. Despite normal binding of NF-κB to DNA, NF-κB-mediated transcription activity was impaired in the presence of caspase-resistant PARP-1 [55]. This study indicated that cleavage of PARP-1 might be required for the late NF-κB-dependent inflammatory response.

Caspase-8 was the most proximal caspase in the caspase cascade and had been known for its role in the mediation of cell death by various death receptors belonging to the TNFR family but also for its non-apoptotic function in T-cell development and differentiation [135].

3.2.6. PARP-1 and disorders

3.2.6.1. PARP-1 and necrotic cell death

Some studies showed that several cell types derived from PARP-1 -/- mice were protected against necrotic cell death [136-138 and reviewed in ref. 115]. These studies clearly suggested that PARP-1 plays an important role in necrotic cell death, which was in sharp contrast to its putative and negligible functions in apoptosis. The induction of PARP-1 enzyme activity by DNA strand breaks or by other stimuli resulted in synthesis of poly(ADP-ribose)polymers by consuming NAD^+ as a substrate. The enzyme activity of PARP-1 is thus expected to affect cellular energy levels. Together, PARP-1 was suggested to play a key role in the processes of cellular energy dynamics and necrotic cell death [136].

According to the ‘PARP-1 suicide’ model, massive levels of DNA damage led to over-activation of PARP-1 which might rapidly deplete intracellular

NAD^+ levels, thereby reducing the rate of glycolysis and electron transport in the mitochondria and abolishing ATP formation, which results in cellular dysfunction and, finally, in necrotic cell death. Inhibition of PARP-1 enzyme activity or complete absence of PARP-1 significantly improved cellular energetics and cell viability after exposure to necrosis-inducing agents [129, 130, 137, 139, 140, and reviewed in refs. 115, 141].

3.2.6.2. PARP-1 and cancer

Initial analysis of cells isolated from PARP-1 -/- mice revealed a measurable genomic instability, although without consequences for the mice, since PARP-1 -/- mice did not show any particular predisposition to develop spontaneous tumors [142-144]. Other studies showed that PARP-1 might have an indirect role in tumorigenesis [145-148]. It was suggested that the elevated tumorigenicity observed in PARP-1 -/- mice under non-physiological conditions might be at least in part due to the transcriptional down regulation of genes involved in cell differentiation or maintenance of genomic integrity and up regulation of the expression of extracellular matrix or cytoskeletal proteins which were implicated in cancer initiation or progression [149]. The exact role of PARP-1 in tumorgenesis is still under debate. It seems to be dependent on the tumor type and genetic background.

3.2.6.3. PARP-1 and inflammatory disorders

The regulation of immune and inflammatory responses is a complex physiological process that is of profound importance to both homeostasis and ultimate survival of an organism. The inflammatory response is composed of an elaborate cascade of inflammatory mediators. It is important to activate both pro- and anti-inflammatory mechanisms in a balanced manner to survive in presence of environmental [4].

A pathophysiological role for PARP-1 had been demonstrated in a number of diseases and animal models, including streptozotocin-induced diabetes [112, 140, 150], zymosan-induced vascular failure, a non-septic model of multiple organ dysfunction [151], LPS-induced septic shock, and carrageen-induced pleurisy [108, 111, 152-153], as well as collagen-induced arthritis (CIA), a model for chronic inflammation [111, 131] (see also Table 2).

Type of disease	Common phenotype of PARP-1 -/- mice
LPS-induced septic shock	full resistance to endotoxic shock
Acute respiratory distress syndrome	reduction of high-permeability pulmonary edema and flooding of alveolar spaces by neutrophilic leukocytes, due to reduced recruitment of polymorphonuclear neutrophils
2,4,6-trinitro-benzene-sulfonic acid-induced mucosal injury in murine colitis	reduction of mucosal injury due to resolution of colonic damage and reduction of neutrophil infiltration; reduction of necrosis in endothelial cells of intestine
Streptozotocin (STZ)-induced diabetes	normoglycemic, reduced sensitivity to SZT and normal function of endothelial and pancreatic β cells
Myocardial postischemic injury	reduction of myocardial infarct size
Hemorrhagic shock	increased survival advantage due to protection from cardiovascular decompensation, vascular hypocontractility, gut barrier failure, and lung neutrophil recruitment
Zymosan-induced inflammation and multiple organ failure (MOF)	resistance against zymosan-induced inflammation and MOF due to reduced neutrophil recruitment and organ injury.

Table 2. Partial list of disease models in which PARP-1 is involved [4].

3.2.6.4. PARP-1 and type I diabetes

Type 1 diabetes is an autoimmune disease characterized by the selective T lymphocyte-mediated destruction of insulin-secreting β cells in the pancreatic islets of Langerhans, which is thought to be the determining event in the pathogenesis of type 1 diabetes [reviewed in refs. 154, 155]. Environmental factors (pathogens, drugs, and diet) and the genetic background (major histocompatibility complex and non-MHC genes) are critical for the initiation of the autoimmune response against the pancreatic β cells [156].

There are two streptozotocin-induced animal models which mimicked at least in part several biochemical and pathological hallmarks of type 1 diabetes in non-human animals [157, 158]. Streptozotocin is a specific β cell toxin which is taken up by β cells through the glucose transporter Glut-2 [159]. Streptozotocin-induced and human autoimmune type 1 diabetes are both characterized by a progressive hyperglycemia and insulitis, associated with drastic up-regulation of the inducible isoform of nitrite oxide (NO) synthase (iNOS) in β cells and islet-infiltrating immune cells [131, 160, 161].

Stimulation of β cells by polymorphonuclear neutrophil (PMN)/ macrophage/T cell-generated cytokines induces the expression of inflammatory mediators, such as iNOS and subsequent massive production of NO and related free radical species such as peroxynitrite. The formation of

free radical species in β cells inhibits insulin secretion, leads to extensive DNA damage and thereby activates the PARP-1 ‘suicide pathway’ which finally results in cellular energy depletion and necrotic β cell death [112, 140, 162, 163].

Type 1 diabetes is also associated with an increase in formation of advanced glycation end product (AGE) in diabetic retinal vessels and renal glomeruli [reviewed in ref. 154]. The activity of AGEs influences two different events: first, they modify extracellular matrix components and intracellular proteins thereby altering their function and, second, certain of these modified plasma proteins bind to AGE receptors on endothelial cells, mesangial cells or macrophages, thereby inducing the activation of the NF-κB [reviewed in refs. 154, 164] causing pathological changes in gene expression of pro-inflammatory mediators [165]. In the last decade, several independent groups have shown that novel inhibitors of PARP enzyme activity not only do provide a significant protection but also PARP-1 -/- mice of different genetic background were nearly completely protected from streptozotocin-induced diabetes [134, 140, 160, 166, 167]. Upon treatment with streptozotocin, PARP-1 -/- mice remained normoglycemic and their β cells showed preserved structure and function concomitant with a drastic reduction in peroxynitrite formation, neutrophil recruitment, and endothelial dysfunction [134, 140, 160, 166, 167].

3.2.6.5. PARP-1 and septic shock

Septic shock is the most common cause of death in intensive care units, with a high mortality rate, often as a result of a systemic gram-negative bacterial infection. It is defined as an acute circulatory failure of dysfunction of a number of organ systems associated with severe sepsis, persisting despite adequate fluid resuscitation, causing a shock-like state and leading go death [4].

Septic shock can be mimicked by intravenous injection of microbial products such as bacterial LPS. LPS activate a complex signaling cascade, enabling the expression of many crucial genes involved in the pathogenesis of septic shock, such as cytokines, adhesion molecules, and iNOS [168]. One of the striking features of septic shock is the increased production of

peroxynitrite which can also induce massive levels of DNA SSBs and activation of the PARP-1 ‘suicide-pathway’ resulting in necrotic cell death and endothelial dysfunction [169-171].

PARP inhibitors could strongly reduce tissue damage caused by high doses of endotoxin [170]. Moreover, PARP-1 -/- mice were extremely resistant to LPS-induced lethality [152, 153]. The production of peroxynitrite and neutrophil recruitment during endotoxic shock as well as local and systemic inflammation were drastically reduced in the absence of PARP-1 [152, 153].

3.3. CARM-1

3.3.1. CARM-1

Coactivator-associated arginine methyltransferase (CARM-1/PRMT-4) was identified as SRC-2/TIF2/GRIP1-binding protein and belongs to a family of arginine-specific protein methyltransferases, which includes at least eight members (PRMT1-8) [55].

CARM-1 had been shown to synergistically enhance transcription by nuclear receptors in combination with the p160 family of coactivators and to form a ternary complex with p300/CBP and SRC-2/TIF2/GRIP1. After recruitment to the promoters of estrogen-responsive genes, CARM-1 methylated specific arginine residues (Arg 17 and Arg26) in the N-terminal tail of histone H3 as part of the transcriptional activation process. Recent studies broadened the targets of the transcriptional coactivator function of CARM-1: CARM-1 coactivated p53-dependent transcription and cooperated with β-catenin to enhance transcriptional activation by the lymphoid enhancer factor/T-cell factor (LEF1/TCF4). Mice with a targeted disruption of CARM-1 die during late embryonic development or immediately after birth, supporting the idea that CARM-1 is a crucial coactivator for gene expression during late embryonic development. Aberrant T-cell development in CARM-1 deficient embryos was due to a partial developmental arrest in the earliest thymocyte progenitor subset, indicating that CARM-1 plays a significant role in promoting the differentiation of early thymocyte progenitors [55].

3.3.2. CARM-1 und NF-κB

The assembly of a higher order NF-κB transcriptional initiation complex is an important stage in NF-κB-dependent transcription, involving multiple coactivator/cofactor-NF-κB-DNA interactions. During this thesis work, coactivator-associated arginine methyltransferase CARM-1/PRMT-4 was described to function as a novel transcriptional coactivator of NF-κB. CARM-1 formed a complex with p300 and NF-κB *in vivo* and interacted directly with the NF-κB subunit p65 *in vitro*. Moreover, CARM-1 synergistically coactivated NF-κB-mediated transactivation, in concert with the transcriptional coactivators p300/CREB-binding protein and the p160 family of steroid receptor coactivators. For at least a subset of NF-κB target genes, the enzymatic activities of both CARM-1 and p300 were necessary for the synergistic activities of CARM-1 and p300 [55].

3.4. Target genes of NF-κB

The induced NF-κB transcription factor promotes the expression of over 500 target genes. The majority of proteins encoded by NF-κB target genes participated in the host immune response [2]. The web page http://people.bu.edu/gilmore/nf-kb/target/index.html shows one possible classification also presented in a simplified manner in Annex A.

3.4.1. Short description of the genes used in these studies

3.4.1.1. House keeping genes

These genes are constitutively expressed in the investigated cells. They were included in the analysis as controls or internal standards for interpretation of the qualitative/semi-quantitative gene expression data (i.e. differences in cellular input, RNA quality, and RT efficiency between samples) [171].

β-Actin

Actin is a protein of the cytoskeleton.

GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acts as an enzyme in the glycolysis pathway.

HPRT

Hypoxanthine ribosyltransferase HPRT is a crucial enzyme involved in the nucleotide metabolism.

3.4.1.2. Cytokines

Cytokines are proteins that regulated cellular interactions, cell growth and secretion. As a result, they regulated many aspects of the immune system.

IL-6

Interleukin-6 (IL-6) is a crucial inflammatory cytokine of 185 amino acids glycosylated at positions 73 and 172. It is synthesized as a precursor protein of 212 amino acids. It exists in different molecular forms. They mainly differ by post-translational alterations such as glycosylation and phosphorylation. In serum IL-6 is complexed with α-2-Macroglobulin, which protected IL-6 from cleavage by proteases. IL-6 is a pleiotropic cytokine influencing antigen-specific immune responses and inflammatory reactions. It is one of the major physiological mediators of acute phase reactions. IL-6 induced B-cell differentiation and activation of T-cells. For instance, in the presence of IL-2, IL-6 induced the differentiation of mature and immature T-cells into cytotoxic T-cells. IL-6 is capable of inducing the final maturation of B-cells into immunoglobulin-secreting plasma cells if the cells had been pre-activated by IL-4. In B-cells IL-6 stimulated the secretion of antibodies to such a degree that serum IgG1 levels could raise 120-400-fold [174].

IL-6 is produced by many different cell types mainly monocytes, fibroblasts, and endothelial cells upon stimulation *in vivo*. Macrophages, T-cells and B-lymphocytes, granulocytes, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cells, and keratinocytes can also produce IL-6 upon stimulation [174].

The human IL-6 gene promoter contains many different regulatory elements allowing the induction of expression by various stimuli. Physiological

and pathophysiological stimuli for the synthesis of IL-6 are PDGF, Ocostatin M, IL-1, bacterial endotoxins, and TNF-α. For instance, in fibroblasts the synthesis of IL-6 is stimulated by IFNβ, TNF-α, PDGF, and viral infections. IL-6 also stimulates or inhibits its own synthesis, depending on the cell type. Macrolide antibiotics spiramycin and erythromycin stimulated the synthesis of IL-6 in human monocytes following cell activation by bacterial lipopolysaccharides. Glucocorticoids or TGF-β inhibit the synthesis of IL-6 depending on the cell type and stimuli [174].

The NF-κB binding site is responsible for the induction of the IL-6 gene expression by IL-1 or TNF-α in non-lymphoid cells. In lymphoid cells, a factor related to the Rel oncogene functioned as a repressor that prevented the interaction of transcription factors with the IL-6-κB binding site [174].

TNF-α

Human tumor necrosis factor alpha (TNF-α) is a non-glycosylated protein of 17 kDa with a length of 157 amino acids. Interestingly, murine TNF-α is N-glycosylated. TNF-α forms dimers and trimers. The 17 kDa form is produced through processing of a precursor protein of 233 amino acids by the TNF-α converting enzyme. TNF-α contains a single disulfide bond that can be destroyed without altering the biological activity of the factor.

TNF-α is secreted by macrophages, monocytes, neutrophils, neutrophilic granulocytes, T-cells and NK-cells following stimulation i.e. bacterial LPS *in vivo* and *ex vivo*. In addition, a number of transformed cell lines, astrocytes, microglial, smooth muscle cells, and fibroblasts can also secrete TNF-α *ex vivo* [174].

The synthesis of TNF-α is induced by many different stimuli including bacterial toxins, interferons, IL-2, GM-CSF, Bradykinin, immune complexes, inhibitors of cyclooxygenase and PAF. The production of TNF-α can be inhibited by IL-6, TGF-β, vitamin D3, prostaglandin E2, dexamethasone, Cyclosporine A and antagonists of PAF [174].

TNF-α causes cytolysis of many cell types including tumor cells [174]. Sensitive cells die within hours after exposure to picomolar concentrations of TNF-α *ex vivo*. The cytotoxic mechanism was shown to involve mitochondria-derived second messenger molecules serving as common mediators of TNF-

α cytotoxic and gene-regulatory signaling pathways [174]. TNF-α induces hemorrhagic necrosis of transplanted tumors *in vivo*. Within hours after injection TNF-α led to the destruction of small blood vessels within malignant tumors. The factor also enhanced phagocytosis and cytotoxicity in neutrophilic granulocytes and modulates the expression of many other proteins, including IL-1 and IL-6. TNF-α induces the synthesis of a number of chemoattractant cytokines, including IP-10, KC, in a cell-type and tissue-specific manner. In addition, TNF-α is a growth factor for normal human diploid fibroblasts. It promotes the synthesis of collagenase and prostaglandin E2 in fibroblasts. In resting macrophages TNF-α induces the synthesis of IL-1 and prostaglandin E2. It also stimulates phagocytosis and the synthesis of superoxide dismutase in macrophages. TNF-α activates osteoclasts and thus induces bone resorption. TNF-α inhibits the synthesis of lipoprotein lipase and thus suppressed lipogenic metabolism in adipocytes. The different activities of TNF-α on various cell types, i.e. growth-promoting and growth-inhibiting activities, were probably mediated by the specific expression pattern and/or regulation of multiple receptors in combination with other distinct receptor-associated proteins [174].

Regarding its role in inflammatory disorders, TNF-α is responsible for some of the severe effects during gram-negative sepsis. On the other hand, TNF-α is required for the B- and T- cell mediated immunity against obligate and facultative bacteria and parasites. It conferres protection against infection with *Listeria monocytogenes*, and antibodies against TNF-α weaken the ability of mice to cope with these infections. Apart from the membrane-bound receptors several soluble proteins that bind TNF-α has been described. These proteins of approximately 30 kDa, called tumor necrosis factor binding protein 1 and 2 (T-BP1 and T-BP2), are similar to the TNF-α-binding domain of the membrane receptor. They can be isolated from urine and serum and probably function as physiological regulators of TNF-α activities by inhibiting binding of TNF-α to its receptor [174].

3.4.1.3. Chemokines

Chemokines are a family of pro-inflammatory and chemotactic cytokines with a characteristic sequence of four cysteine residues. They regulate the migration of leukocytes from blood into tissues.

IP-10

Interferon-inducible protein-10 (IP-10) has a length of 98 amino acids and shows homology to platelet factor-4 (PF4).

The expression of IP-10 is induced by LPS, IFNγ and/or TNF-α in a variety of cells including monocytes, endothelial cells, keratinocytes and fibroblasts. Human neutrophils produce IP-10 in response to IFNγ in combination with either TNF-α or LPS and this response is blocked by IL-10 and IL-4. IFNγ alone or in association with agonists such as IL-8 and GM-CSF has no effect. However, IFNγ in combination with TNF-α, LPS, or IL-1β, resulted in a considerable induction of IP-10 release by neutrophils, *ex vivo* [174].

IP-10 had been detected in keratinocytes, lymphocytes, monocytes, and endothelial cells in immunologically mediated processes. Keratinocytes in normal epidermis did not produce IP-10. Murine IP-10 is synthesized predominantly in the liver and kidney after intravenous injection of inflammatory agents, and in particular after injection of IFNγ, and thus, might play an important role in the response of liver and kidney to systemic inflammation. IP-10 mRNA expression was more sensitive to suppression by IL-4 when stimulated by bacterial LPS than by IFNγ/IL-2 [174].

IP-10 probably also plays a role in regulation of the growth of immature hematopoietic progenitor cells and has been shown to be a potent endogenous inhibitor of angiogenesis [174].

KC

Keratinocyte derived chemokine (KC) was initially identified by its overexpression in murine monocytes and macrophages in response to PDGF and M-CSF treatment. KC was an early response gene. Its synthesis was also induced by TNF-α. KC was involved in neutrophil chemotaxis and activation [174].

M-CSF

Macrophage chemotactic colony stimulating factor (M-CSF) was a homodimeric glycoprotein linked by disulfide bonds. The sugar moiety is not required for the full spectrum of biological activities. Different molecular forms of M-CSF with lengths between 256 and 438 amino acids have been described. They arise by translation of alternatively spliced mRNAs. M-CSF-β is a secreted form that occurs not in a membrane-bound form. M-CSF-α is expressed as an integral membrane protein that is slowly released by proteolytic cleavage. The membrane-bound form of M-CSF interacts with receptors on near-by cells and therefore mediates specific cell-to-cell contacts. Additionally, some high-molecular weight forms of murine M-CSF have been described. These forms are complexed with proteoglycan of the intracellular matrix [174].

M-CSF is produced *ex vivo* and *in vivo* by monocytes, granulocytes, endothelial cells and fibroblasts. After cell activation, B-cells, T-cells and also a number of tumor cell lines are also capable of synthesizing this factor. Moreover, M-CSF had been found to be synthesized by uterine epithelial cells *in vivo*. The factor was found also in human urine [174].

M-CSF was initially isolated as a factor stimulating the growth of macrophage/granulocyte-containing colonies in soft agar cultures. M-CSF influences the proliferation and differentiation of hematopoietic stem cells into macrophages. In combination with another colony stimulating factor, GM-CSF, the phenomenon of synergistic suppression could be observed. M-CSF is highly specific colony stimulating factor and more or less restricted to the macrophage lineage. In human macrophages M-CSF induced antibody-dependent cellular cytotoxicity [174].

IL-1, TNF-α, IFNγ, GM-CSF and PDGF could induce the synthesis of M-CSF. Prostaglandins, glucocorticoids and substances that raise intracellular levels of cAMP inhibit the synthesis of M-CSF. In monocytes and macrophages M-CSF induces the synthesis of IL-1, G-CSF, IFNγ, TNF-α, plasminogen activator, thromboplastin, prostaglandins and thromboxanes and also oxidative metabolism. M-CSF could act synergistically with IL-1, IL-3 and IL-6 in the stimulation of proliferation and the differentiation of primitive hematopoietic cells into macrophages [174].

MIP (MIP-1 and MIP-2)

Macrophage inflammatory proteins 1 and 2 (MIP-1 and MIP-2) are the major factors produced by macrophages following their stimulation with bacterial endotoxins [174].

MIP-1α and MIP-1β are acidic proteins with a length of 69 amino acids that corresponded to 7.8kDa. Their cDNAs share a homology of 57 percent and at the protein level a homology of 60 percent. Both proteins are involved in the cellular activation of human granulocytes (neutrophils, eosinophils and basophils) and appear to be involved in acute neutrophilic inflammation. Both MIP-1α and MIP-1β stimulate the production of reactive oxygen species in neutrophils and the release of lysosomal enzymes. They also induce the synthesis of other proinflammatory cytokines such as IL-1, IL-6 and TNF-α in fibroblasts and macrophages [152]. MIP-1α is a potent basophil agonist, inducing a rapid change of cytostolic free calcium, the release of histamine and sulfido-leukotrienes, and chemotaxis. Murine MIP-1α is the primary stimulator of TNF-α secretion by macrophages, whereas MIP-1β antagonizes the inductive effects of MIP-1α. In human monocytes the production of MIP-1β is induced by bacterial lipopolysaccharides and IL-7. MIP-1α and MIP-1β can also act synergistically with hematopoietic growth factors. Both MIP-1α and MIP-1β enhance the activities of GM-CSF and promoted the growth of more matur hematopoietic progenitor cells. MIP-1α (but not MIP-1β) also acts as an inhibitor of the proliferation of immature hematopoietic stem cells. MIP-1α have been shown to be capable of protecting hematopoietic progenitor cells from the cytotoxic effects of the cell cycle-specific drug hydroxyurea *in vivo* and might also be of clinical significance [174].

MIP-2 is a basic protein of approximately 6 kDa. It is extremely chemotactic for segmented neutrophilic granulocytes and acts also synergistically with GM-CSF and M-CSF. In contrast to MIP-1, MIP-2 induces also the degranulation of human neutrophils but did not enhance oxidative metabolism [174].

MCP-1

Monocyte chemoattractant protein-1 (MCP-1) is produced *ex vivo* by monocytes, vascular endothelial cells, smooth muscle cells, glomerular

mesangial cell, osteoblastic cells and human pulmonary type-2-like epithelial cells. Its expression is induced by LPS but not by TNF-α. In addition, IgG complexes could rapidly induce the synthesis and release of MCP-1 in mesangial cells [174].

MCP-1 is chemotactic for monocytes but not neutrophils. It regulates the expression of cell surface antigens and the expression of cytokines such as IL-1 and IL-6. MCP-1 is a potent activator of basophils, inducing the degranulation and the release of histamines. In basophils activates MPC-1 enhanced the synthesis of leukotriene C4. MCP-1 has also been shown to exhibit biological activities other than Chemotaxis. It could induce the proliferation and activation of killer cells [174].

RANTES

RANTES is the abbreviation of regulated upon activation, normal T-cell expressed, and presumably secreted. RANTES is an early response gene and its synthesis was induced by LPS, TNF-α, but not by IL-6 [174].

RANTES is preferentially highly expressed in T-cells but also moderately in other cell types. RANTES is chemotactic for T-cells, human eosinophils and basophils and plays an active role in recruiting leukocytes into inflammatory sites. It changes the density of eosinophils and makes them hypodense, which is thought to represent a state of cell activation. RANTES was also a potent eosinophil-specific activator of oxidative metabolism. RANTES could increase the adherence of monocytes to endothelial cells. It selectively supported the migration of monocytes and T-lymphocytes expressing the cell surface markers CD4 [174].

3.4.1.4. Adhesion molecules

ICAM-1

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin gene superfamily, which bound to several surface molecules and was involved in the initiation of the immune reaction [174].

ICAM-1 is induced by cytokines and various stress stimuli, such as hypoxia, ultraviolet, and ionizing radiation [174].

3.4.1.5. Enzymes

COX-2

Cyclooxygenase-2 (COX-2) is a member of the COX family and catalyses the first committed step in the synthesis of prostanoids, which represents a large family of arachidonic acid metabolites comprising prostaglandins, prostacyclin, and thromboxanes [174].

The Cyclooxygenase family is a major target of non-steroidal anti-inflammatory drugs (NSAIDs). COX exists as constitutive (COX-1 and COX-3) expressed gene products and as an inducible protein (COX-2). COX-2 is rapidly expressed in several cell types in response to growth factors, cytokines, and pro-inflammatory molecules. Since its discovery in the early 1990s, COX-2 had emerged as a major player in inflammatory reactions in peripheral tissues [174].

iNOS

Inducible nitric oxide synthase (iNOS) is a member of the NOS family including endothelial NOS (eNOS), neuronal NOS (nNOS) and iNOS. The NOS family of enzymes catalyzes the conversion of L arginine to the cytostatic molecule nitric oxide (NO). eNOS and nNOS are constitutively expressed and are regulated by posttranslational modification [174].

The inducible NOS (iNOS) is an early response gene induced by an acute inflammatory insult or mechanical perturbation. Once expressed, the iNOS activity is generally limited only by the substrate availability [174].

3.4.1.6. Receptors of the immune system and NF-κB signaling cascade

TLR (TLR-2 and TLR-4)

Toll-like receptors (TLRs) belong to a large family (TLR1-15). TLRs are transmembrane receptors mainly involved in the pathogen pattern recognition but also partially involved in developmental processes. TLR proteins are characterized by an extracellular leucine-rich repeat domain and a cytoplasmic IL-1 receptor-like region known as the TIR domain (Toll-IL-1 receptor domain) [174].

The receptors highly specifically recognize the invasion of distinct microorganisms and play the key role in innate immune responses. Distinct TLR receptors bind bacterial cell wall components, viral and bacterial oligonucleotides (RNA & DNA), thereby activating innate immune responses and subsequently at least in part adaptive immunity. Engagement of these receptors activates transcription factor NF-κB, which initiates the transcription of genes encoding pro-inflammatory cytokines [174].

TLR-2 and TLR-4 are the 2 major TLRs in mammals. TLR-2 is highly expressed in peripheral blood leukocytes but also moderately in heart, brain, and muscle. Signaling through TLR-2 in mammalian cells involves activation of NF-κB. In contrast to TLR-4, TLR-2 expression is upregulated by bacterial LPS only in granulocytes but not in monocytes. This expression is not affected by IL-10. -/- mice lacking TLR-2 are highly susceptible to infection with *Staphylococcus aureus* compared to wild type (+/+) mice. Macrophages from such mice produced only low levels of TNF-α and IL-6. TLR-2 appears to be a molecular link between microbial products, apoptosis, and host defense mechanisms since induction of IL-12 production by microbial lipoproteins in human macrophages is also mediated by other TLRs. Microbial lipoprotein-mediated apoptosis however is only signaled through human TLR-2 in these cells [174].

Signaling through TLR-4 involves also the activation of NF-κB in mammalian cells. TLR-4 expression is induced by bacterial lipopolysaccharides and predisposes animals to the development of gram-negative sepsis. -/- mice lacking TLR-4 were described to be highly resistant to LPS-induced septic shock [174].

IRAK-4

IL-1 receptor-associated kinase-4 (IRAK-4) is a mediator of signaling through the IL-1 receptor and TLRs. The protein has an N-terminal death domain and a central kinase domain. An IRAK-4 isoform lacking the kinase domain inhibits activation of NF-κB and IRAK-1 by IL-1, but not by TNF-α. IRAK-4 plays an essential role in pathogen pattern recognition signaling cascades [174].

IκBα

see the chapter 3.1.2.

3.5. Aim of the thesis

Aim of this thesis was to investigate NF-κB-dependent genes in regard to their depending on PARP-1 or CARM-1, respectively. Additional the particular contribution of the enzymatic PARP activity and caspase-mediated cleavage of PARP-1 on NF-κB-dependent gene expression was analyzed. For this purpose, wild-type (+/+) cells, untreated or treated with the corresponding inhibitors, were compared with the corresponding knock-out (-/-) or knock-in (KI/KI) cells. 16 genes were chosen from over 500 target genes of NF-κB. PJ-34 and zVAD-fmk were used as inhibitors of PARP-1 and caspases, respectively.

Several time points were included in this study to obtain semi-quantitative kinetics of NF-κB-dependent gene expression. The project of this thesis was divided in three parts:

1. Established of RT-PCR conditions for each single pair of RT-PCR primers (described in the Method and Material part).
2. Identification of PARP-1 and CARM-1 dependent target genes of NF-κB
3. Identification of PARP-1 dependent target genes of NF-κB, which are also regulated by enzymatic activities either of PARPs or caspases.

4. Results

4.1. Target genes of PARP-1

In order to test which subsets of NF-κB-dependent genes are impaired in primary PARP-1 -/- cells primary mouse lung fibroblasts freshly isolated from PARP-1 +/1 and -/- mice were treated with LPS as indicated and the expression of NF-κB-dependent genes was assessed by reverse transcription-PCR (see Figure 6).

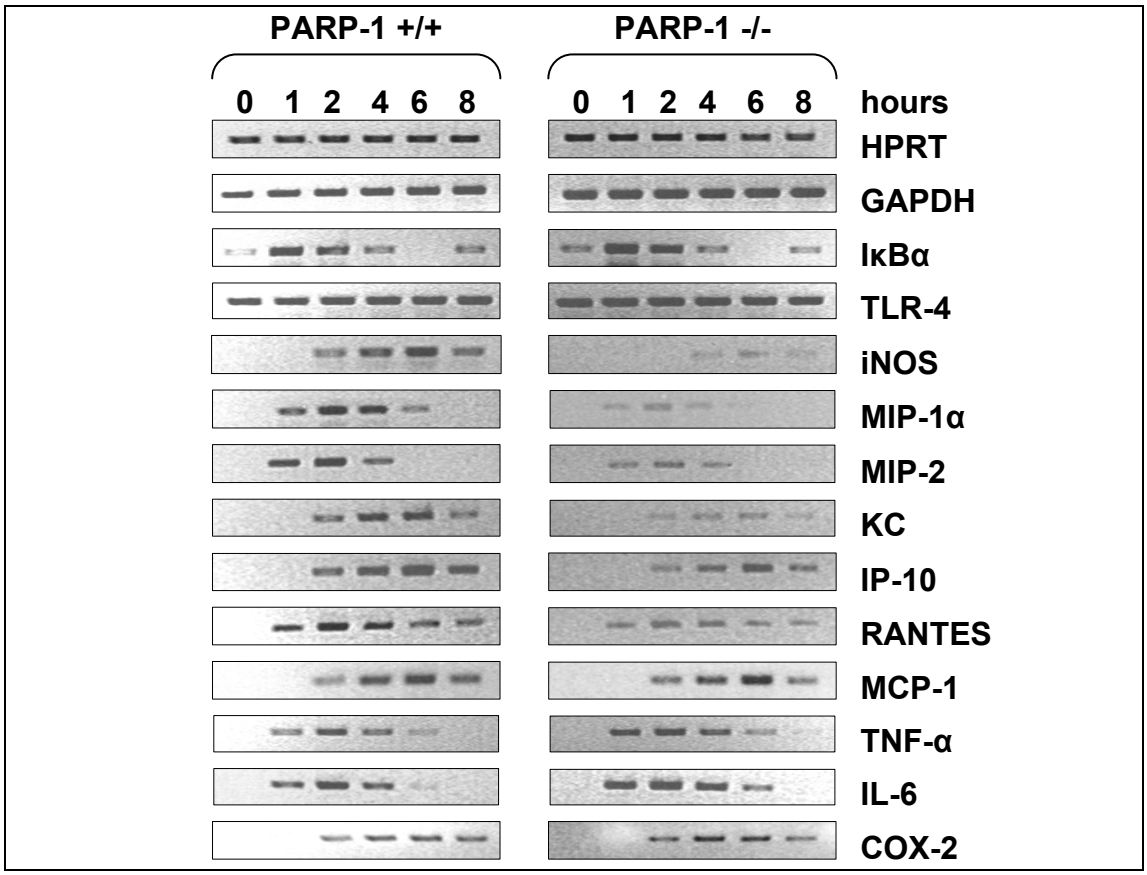


Figure 6. Impaired expression of iNOS, MIP-1α, MIP-2, KC, IP-10 and RANTES in PARP -/- cells in response to proinflammatory stimuli. PARP-1 +/+ and PARP-1 -/- MLFs were treated with 1μg/ml LPS and RNA isolated at the indicated time points, followed by RT−PCR determination of HPRT, GAPDH, IκBα, TLR-4, iNOS, MIP-1α, MIP-2, KC, IP-10, RANTES, MCP-1, TNF-α, IL-6 and COX-2 mRNA. mRNA levels were normalized to the HPRT and GAPDH mRNAs.

The experiments revealed that LPS-induced levels of iNOS, MIP-1α, MIP-2 and KC were strongly reduced in PARP-1 -/- cells whereas the levels of IP-10 and RANTES were only moderately reduced in these cells. Additionally, the expression of IκBα, TNF-α, IL-6 and COX-2 was not impaired in PARP-1

-/- cells. The mRNAs of IL-6 and COX-2 were even slightly upregulated in PARP-1 -/- cells.

4.2. Target genes of non-cleaved and cleaved PARP-1

In order to test whether cleavage of PARP-1 might be required for early NF-κB-dependent transcription, primary mouse lung fibroblasts freshly isolated from PARP-1 +/+ and DEVD(214)-KI/KI mice were treated with LPS as indicated and the expression of NF-κB-dependent genes was assessed by reverse transcription-PCR (see Figure 7).

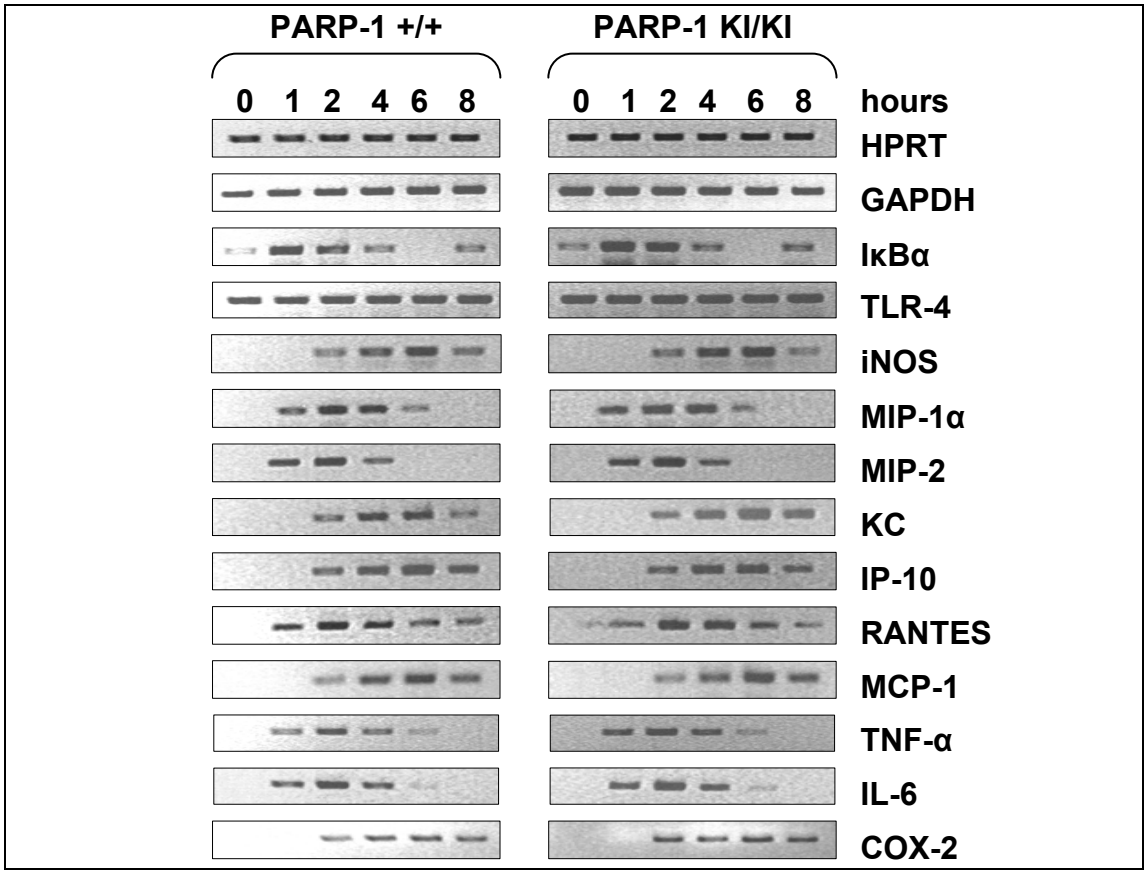


Figure 7. PARP-1 +/+ and PARP-1 KI/KI MLFs were treated with 1μg/ml LPS and RNA isolated at the indicated time points, followed by RT−PCR determination of HPRT, GAPDH, IκBα, TLR-4, iNOS, MIP-1α, MIP-2, KC, IP-10, RANTES, MCP-1, TNF-α, IL-6 and COX-2 mRNA. mRNA levels were normalized to the HPRT and GAPDH mRNAs.

The experiments revealed that no tested NF-κB-dependent genes were downregulated in PARP-1 KI/KI cells at early time points of the inflammatory response, indicating that cleavage of PARP-1 might be only required for the second wave of NF-κB-dependent inflammatory response.

It was recently shown by several independent groups that Caspase-8 could activate the NF-κB pathway independent of its activity as a pro-apoptotic protease. Caspase-8 was identified as an essential component in the activation of the IKK complex, linking IKK to the upstream Bcl10-MALT1 complex. Intriguingly, one study suggested that it was not only the physical presence of caspase-8, but rather its enzymatic activity was required for the activation of NF-κB. It was previously shown that PARP-1 could be cleaved *in vitro* by caspase-8 [175-177].

To confirm these data and to test whether NF-κB-dependent inflammatory response required caspase activities, macrophage-like Raw264 cells were treated with LPS in presence or absence of the caspase inhibitor zVAD-fmk as indicated, and the expression of NF-κB-dependent genes was assessed by reverse transcription-PCR (see Figure 8).

Remarkably, this experiment revealed that expression levels of iNOS, KC, MIP-2, MIP-1α, RANTES and IP-10 were strongly reduced in presence of zVAD-fmk, during the second wave of the NF-κB-dependent inflammatory response, indicating that a subset of NF-κB target genes were dependent on caspase activities. Interestingly, all genes affected by the caspase inhibitor zVAD-fmk were also dependent on PARP-1.

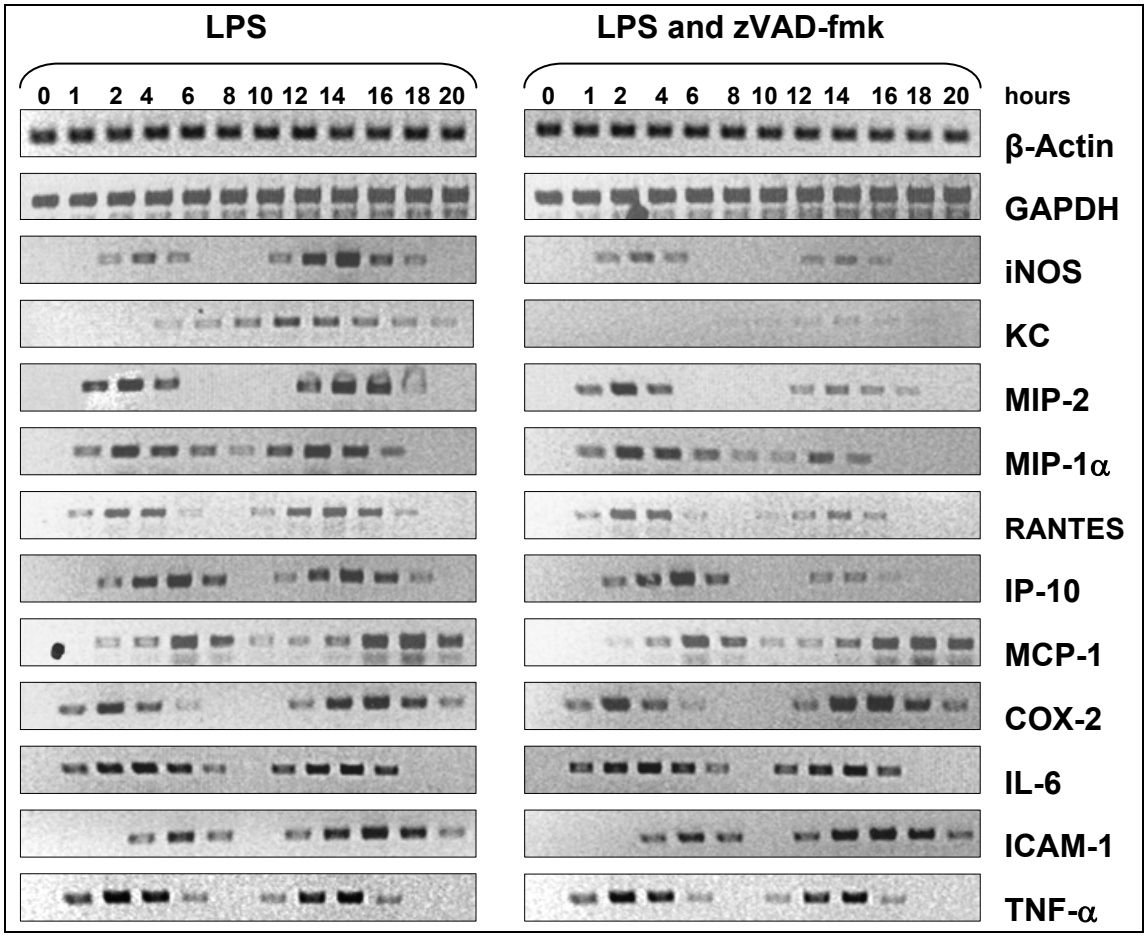


Figure 8. Impaired expression of iNOS, KC, MIP-2, MIP-1α, RANTES and IP-10 in Raw264 cells in presence of the caspase inhibitor zVAD-fmk. Raw264 cells were treated with 0.1µg/ml LPS in presence and absence of 20µM zVAD-fmk and RNA isolated at the indicated time points, followed by RT−PCR determination of β-Actin, GAPDH, IκBα, ICAM-1, iNOS, MIP-1α, MIP-2, KC, IP-10, RANTES, MCP-1, TNF-α, IL-6 and COX-2 mRNA. mRNA were normalized to the HPRT and GAPDH mRNAs.

4.3. PARP-1 Inhibitor

Initial reports using either PARP inhibitors or genetic approaches demonstrated that the enzymatic activity of PARP-1 was not required for NF-κB-dependent transcription. However, other studies recently suggested that drug-mediated PARP inhibition could affect NF-κB-dependent transcription *in vivo*, most likely in a cell type-specific manner [reviewed in ref. 4]. For instance, PARP inhibition with the phenanthridinone-based PARP inhibitor PJ-34 suppressed inflammatory cell migration of macrophages [108]. These effects were associated with down regulation of the CC chemokine MIP-1α, but not the CXC chemokine MIP-2. The production of TNF-α and IL-12, but not IL-5 or IL-13, was also suppressed by PARP inhibition in macrophages

[108].

In order to test whether inhibition of the PARP enzymatic activity affected NF-κB-dependent transcription in macrophages Raw264 cells were treated with LPS in presence or absence of the PARP inhibitor PJ-34 (as indicated), and the expression of NF-κB-dependent genes was assessed by reverse transcription-PCR (see Figure 9).

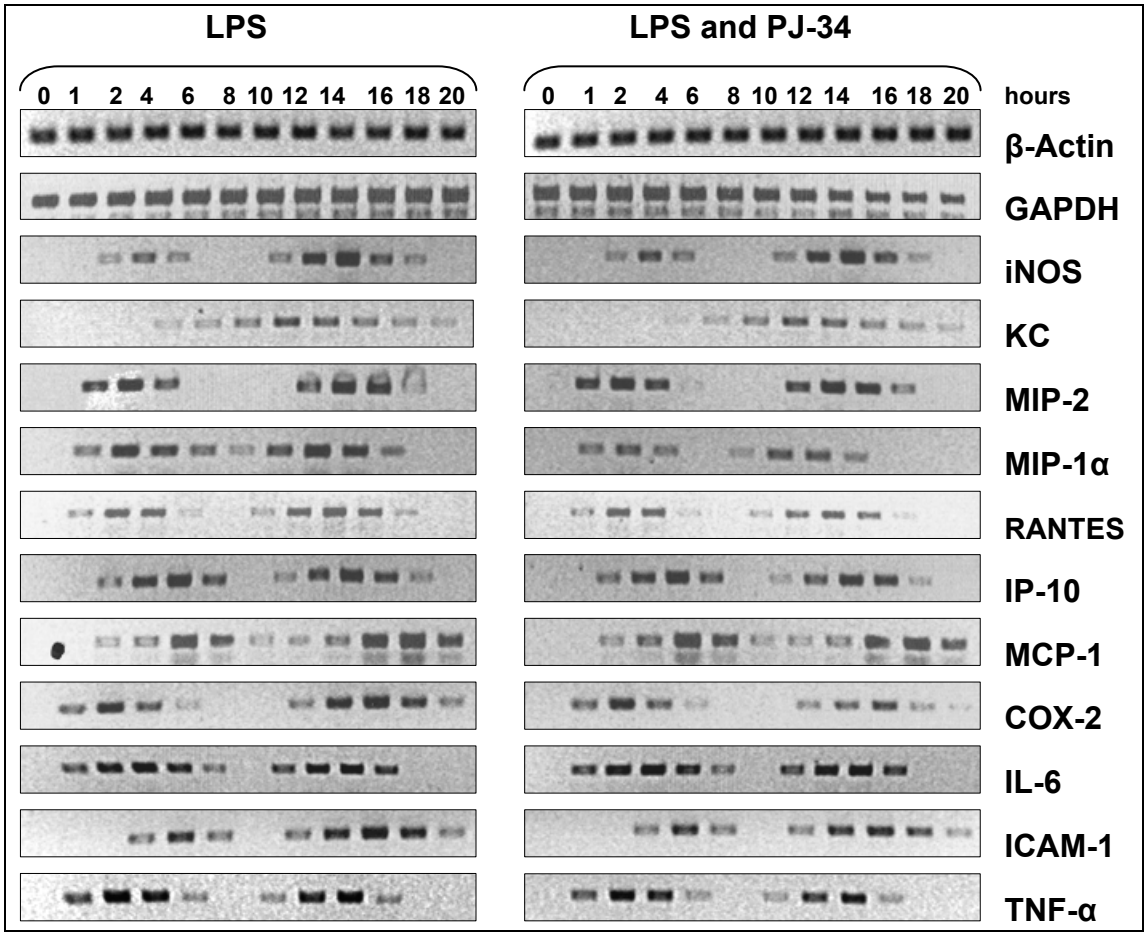


Figure 9. The enzymatic activity of PARP-1 was not required for NF-κB-dependent transcription. Raw264 cells were treated with 0.1μg/ml LPS in presence and absence of 50μM PJ-34 and RNA isolated at the indicated time points, followed by RT−PCR determination of β-Actin, GAPDH, IκBα, ICAM-1, iNOS, MIP-1α, MIP-2, KC, IP-10, RANTES, MCP-1, TNF-α, IL-6 and COX-2 mRNA. mRNA levels were normalized to the HPRT and GAPDH mRNAs.

Strikingly, this experiment clearly demonstrated that except for COX-2, none of tested NF-κB-dependent genes required the enzymatic activity of PARP-1. A significant reduction was observed only for COX-2 in presence of PJ-34 during the second wave of NF-κB-dependent transcription.

4.4. Target genes of CARM-1

Experimental data obtained in our lab strongly indicated that CARM-1 could act as novel coactivator of NF-κB. CARM-1 directly bound to the NF-κB subunit p65 and synergistically coactivated NF-κB-mediated transactivation, in concert with the transcriptional coactivators p300/CBP and the p160 family of steroid receptor coactivators [56].

To investigate which subset of NF-κB-dependent genes was influenced by CARM-1, mouse embryonic fibroblasts (MEFs) isolated from CARM-1 +/+ or CARM-1 −/− mice were treated with LPS (see Figure 10) or TNF-α (see Figure 11 as indicated) and the expression of NF-κB-dependent genes assessed by RT−PCR. These experiments revealed that LPS-induced expression levels of G-CSF, MIP-2, MCP-1, IP-10 and ICAM-1 were impaired in CARM-1 −/− cells during the first wave. But, the same genes were not impaired during the second wave of NF-κB-dependent gene expression (see Figure 10A and B). The slight reduction observed for IP-10 and MCP-1 during the second wave seemed not to be significant enough.

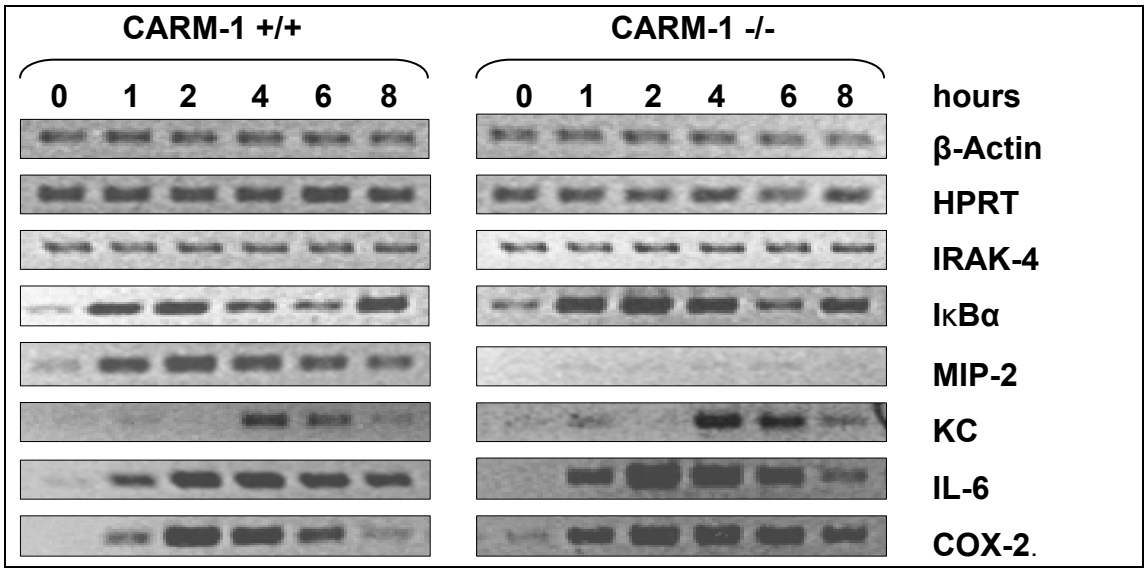


Figure 10A.

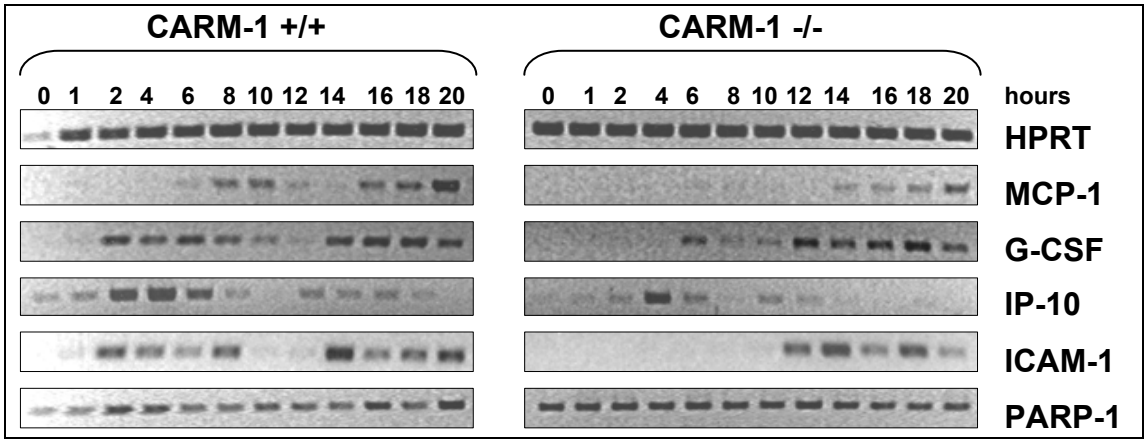


Figure 10B.

Figure 10. Impaired NF-κB-dependent gene expression in CARM-1 -/- cells in response to LPS. CARM-1 +/+ and CARM-1 -/- MEF cells were treated with LPS (10μg/ml) and RNA isolated at the indicated time points, followed by RT−PCR determination (A; first wave only) of IRAK-4, IκBα, MIP-2, IL-6, KC, COX-2, HPRT and β-Actin mRNA or (B; first and second wave) of MCP-1, G-CSF, ICAM1, IP-10, PARP-1 and HPRT mRNA. mRNA levels were normalized to the HPRT and/or β-Actin mRNAs.

Similar results were obtained when cells were stimulated with TNF-α (see Figure 11). However, the expression of IκBα, IRAK-4, TLR-2, TLR-4, IL-6, KC and COX-2 was not reduced, indicating that only a subset of NF-κB-dependent genes require CARM-1 for induction. The expression of COX-2 and IL-6 was even slightly upregulated in CARM-1 −/− cells.

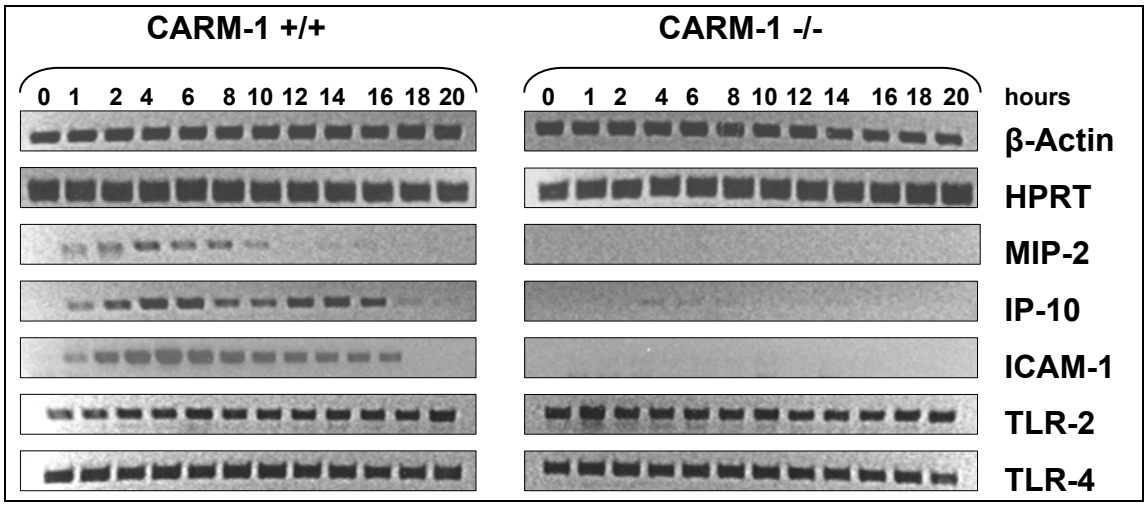


Figure 11. Impaired NF-κB-dependent gene expression in CARM-1 -/- cells in response to TNF-α. CARM-1 +/+ and CARM-1 -/- MEF cells were treated with TNF-α (10ng/ml) and RNA isolated at the indicated time points, followed by RT−PCR determination of MIP-2, IP-10, ICAM-1, TLR-2 TLR-4, HPRT and β-Actin mRNA. mRNA levels were normalized to the HPRT and/or β-Actin mRNAs.

Taken together, these experiments suggested that CARM-1 was required for the expression of a subset of target genes of NF-κB upon stimulation with TNF-α or LPS. Interestingly, CARM-1 seemed to act preferentially during the first wave of NF-κB-dependent gene expression.

4.3. Original publications

Some of the data of this thesis were published with the permission of the dean (see Annex B).

5. Discussion

Virtually all cell types induce NF-κB responses. The activity of NF-κB is regulated at multiple levels: the level of translation, the existence of at least 12 different NF-κB dimers, the interaction of these dimers with specific IκBs and their subcellular localization, posttranslational modification of these dimers in the cytoplasm and the nucleus, differential accessibility of κB sites in various promoter and enhancer, differential binding to κB's response elements due to different affinities, and cell type and stimuli specific interaction with a combination of coactivators. Aim of this thesis was to investigate the dependence of defined NF-κB-dependent genes on PARP-1 or CARM-1, respectively. Additionally, the potential contribution of the enzymatic activities of PARPs and caspase-mediated cleavage of PARP-1 on NF-κB-dependent gene expression were investigated.

5.1. PARP-1 and CARM-1 act as promoter-specific coactivators of NF-κB

Several NF-κB-dependent genes has been identified that were strongly dependent on PARP-1, CARM-1 or caspase induced cleavage of PARP-1. Interestingly, most of the identified target genes were dependent either by PARP-1 or CARM-1. Only two NF-κB-dependent genes, namely MIP-2 and IP-10 seemed to require the presence of both PARP-1 and CARM-1. However, one could not exclude the possibility that the particular requirement was cell type-specific. Table 3 summarizes the identified down or upregulated NF-κB-dependent genes in the corresponding -/- cells.

	PARP-1 -/-, LPS stimulated	CARM-1 -/-, LPS stimulated	CARM-1 -/-, TNF-α stimulated
down-regulated	MIP-2 IP-10 KC MIP-1α RANTES iNOS	MIP-2 IP-10 MCP-1 G-CSF ICAM-1	MIP-2 IP-10 MCP-1
slightly up-regulated	COX-2 IL-6 TNF-α	COX-2 IL-6 IκBα KC	IκBα

Table 3. A summary of identified PARP-1 and CARM-1 target genes

The exact molecular mechanism underlying the promoter-specific activities of PARP-1 and CARM-1 had yet to be investigated. CARM-1 is a classical histone modifier and might therefore modulate NF-κB-dependent gene expression through its enzymatic activity and specific association with NF-κB-dependent promoters. Remarkably, experiments preformed in parallel to this thesis study in the same lab revealed that CARM-1 acted in a gene-specific manner mainly by enhancing NF-κB/p65 recruitment to its cognate sites in the promoter or enhancer region of the corresponding NF-κB target gene. Moreover, CARM-1 formed a complex with p300 and NF-κB *in vivo* and interacted directly with the NF-κB subunit p65 *in vitro*. CARM-1 synergistically coactivated NF-κB-mediated transactivation, in concert with the transcriptional coactivators p300/CREB-binding protein and the p160 family of steroid receptor coactivators. Interestingly, for at least a subset of CARM-1-dependent NF-κB target genes, the enzymatic activities of both CARM-1 and p300 were necessary for the observed synergy between CARM-1 and p300. Given these additional observations, the results of the thesis suggested that the cooperative action between protein arginine methyltransferases and protein lysine acetyltransferases regulates NF-κB-dependent gene activation *in vivo* [55].

A slightly different situation might be the case for PARP-1. Although it was previously shown that PARP-1 could directly bind to both subunits of NF-κB (p50 and p65) and could act as a coactivator of NF-κB in concert with the classical NF-κB coactivator p300/CBP its not yet cleared whether PARP-1 facilitates the recruitment of p65 or p50 to its cognate sites in the promoter or enhancer region of a corresponding NF-κB target gene. Moreover, unlike

CARM-1, PARP-1 is an architectural cofactor and not a classical histone modifier. PARP-1 was initially identified as an active component of an upstream stimulatory fraction from Hela nuclear extracts termed USA cofactor complex. Earlier studies showed that the USA complex is essential in the presence of a complete set of general transcription factors for transcriptional activity of transcription factors such as NF-κB, including p50 homodimers, SP1, Oct-1 and USF [reviewed in ref. 4]. Subsequent fractionation studies of the crude precursor USA fraction in the last decade, led to the discovery of at least 6 independent subfractions of positive cofactors: PC-1/PARP-1, PC-2 ("Mediator" like complex), PC-3 (Dr2/topoisomerase I), PC4 (ssDNA binding protein), PC-5 and PC-6. Several previous studies showed that the cofactor/coactivator function of PARP-1 was not dependent on damaged DNA or on the enzyme activity of PARP-1. Since PARP-1 is a component of the USA derived positive cofactor complex PC-1, PARP-1 might therefore not only function in concert with p300/CBP and potentially CARM-1 (i.e. on MIP-2 and IP-10 promoter) but also synergistically with other USA-derived positive cofactor complexes in stabilizing the interaction between NF-κB and the basal transcription machinery, thereby facilitating the formation and subsequent activation of the preinitiation complex (PIC) *in vivo* [reviewed in ref. 4].

5.1.2. Caspase activities but not the enzymatic activity of PARP are required for NF-κB-dependent gene expression.

Another interesting aspect is the possible involvement of possible poly(ADP-ribosyl)ation activity in NF-κB-dependent gene expression. Several reports had described an inhibitory effect of PARP inhibitors on the expression of other κB-dependent genes in mice [reviewed in ref. 4]. However, while the complete absence of PARP-1 abolished the induction of these NF-κB dependent genes in PARP-1 -/- mice, only a slight inhibition (15 to 40%) was observed when PARP-1 +/+ mice or cells were treated with high doses of nonspecific PARP inhibitors. Moreover, the production of cytokines was not affected in a MDML model of diabetes by PARP inhibitors. Nevertheless, it was suggested that the partial reduction was due to inhibition of the putative chromatin modifying and remodeling activity of PARP-1 [reviewed in ref. 4]. It had been proposed that polynucleosomes could be

completely decondensed upon poly(ADP-ribosyl)ation by PARP-1 *in vivo*, and this in turn might result in full activation of NF-κB-dependent genes. Indeed, the modulation of chromatin structure through histone modification is a very important process during DNA transcription. Histones are dynamically modified during activation of gene expression. Modifications include acetylation, phosphorylation, ubiquitination, methylation, and potentially ADP-ribosylation [reviewed in ref. 178].

However, the data of this thesis, obtained with the broad range non-specific PARP inhibitor PJ-34, do not confirm that the enzymatic activity of PARP-1 is required for NF-κB-dependent gene expression, *ex vivo*. With the potential exception of MIP-1α and COX-2, which were slightly down regulated in presence of PJ-34, no other gene tested was affected by PJ-34. Therefore the slight but still significant inhibitory effect observed *in vivo*, using different mice models could be explained by the observation that certain “messenger molecules” released from necrotic cells, such as HSP70 or HMGs, often induce the activation of NF-κB-dependent gene expression in surrounding healthy cells, as a kind of secondary inflammatory response. Since the enzymatic activity of PARPs seemed to be crucial for the execution of cell death, inhibition of PARPs would not only block necrosis or related cell death processes but also block the secondary inflammatory response.

On the other hand a very interesting observation had been made upon treatment of macrophages with LPS in presence of the broad range caspase inhibitor zVAD-fmk. These experiments revealed that the second gene expression waves of iNOS, KC, MIP-2, RANTES, MIP-1α (only slightly) and IP-10 seemed to be dependent on the enzymatic activity of caspases

It had been recently discovered that Caspase-8 could activate the NF-κB-pathway either dependent or independent of its activity as a pro-apoptotic protease [154-156]. Caspase-10 and MRIT, two human homologs of Caspase-8, could similarly activate the NF-κB-pathway. Dominant-negative mutants of the Caspase-8 prodomain could block NF-κB-induced by Caspase-8, FADD and several death receptors belong to the TNFR family. Caspase-8 interacts with multiple proteins known to be involved in the activation of the NF-κB-pathway, including the serine-threonine kinases RIP, NIK, IKK1 and IKK2 [176, 177]. Moreover c-FLIP(L) was recently shown to influence effector

T-cell function through its activation of caspase-8, which in turn cleaves c-FLIP(L) to allow RIP1 recruitment and NF-κB-activation. This mechanism was suggested to be partially required to initiate proliferation of resting T-cells [158]. Since PARP-1 is also a substrate for Caspase-8 and Caspase-10, the observed down regulation of NF-κB-dependent genes during the second wave of expression might directly depend on PARP-1 cleavage. Indeed, it was previously found that NF-κB-transcriptional activity was impaired in PARP-1 (D214N-KI/KI) mice in response to inflammatory stress [135]. Interestingly, since PARP-1 was fully enzymatic active in PARP-1 KI/KI mice and enzyme inactive PARP-1 could restore NF-κB-activity in PARP-1 –/– macrophages, the enzymatic activity of PARP-1 seems to be dispensable for NF-κB-dependent gene expression. Finally, consistent with the observation that the activation of caspases could occur rapidly *in vivo*, it was reported that caspase inhibitor Z-VAD-fmk and noncleavable PARP-1 showed a protective effect in ischemia/reperfusion (I/R) models [174]**.** These findings were reminiscent of previous studies showing that caspase inhibitors attenuated the mouse response to septic shock, focal and renal I/R, and allergic airway inflammation in the asthma model. Taken together, our RT-PCR data and published data from others provide strong evidence that caspase-mediated PARP-1 cleavage might be required for NF-κB-transcriptional activity, especially for the second wave of NF-κB-dependent gene expression. Since PARP-1 fragments could costimulate NF-κB-activity, it is reasonable to speculate that the cleaved fragments of PARP-1 might modulate the interaction of p300 and NF-κB with the basal transcription machinery. However, the exact molecular mechanisms have yet to be investigated.

5.2. Cautionary notes

5.2.1. Do cell culture systems reflect the real situation *in vivo*?

All data observed in this thesis as well as most of the published data were obtained with *ex vivo* experiments using isolated cells. There are only few studies published that investigated these aspects *in vivo* using animal models. Moreover, most if not all of these *ex vivo* cell culture studies were performed with a single cell type. Additionally, it was known that the phenotypes of primary cells (i.e. PARP-1 +/+ and -/-) are different from those of immortalized cells (i.e. Raw264.1 or CARM-1 +/+ and -/-). It is obvious that the situation is more complex and might be even completely different *in vivo*. Different types of cells in certain tissue were embedded in a special microenvironment, communicated with and influenced each other and therefore might have reacted differentially upon stimulation with distinct sets of stimuli. The transcriptional activity of NF-κB is in a living organism not only dependent on the availabilities, combined actions and interactions of distinct transcriptional coactivator complexes and cofactors but also depends on the interplay of different combinations of stimuli and the signaling between distinct cell types. To obtain the most accurate profile of gene expression patterns *in vivo*, it is absolutely essential to use animal models for genome wide RT-PCR or micro array analysis and immediately isolated distinct types of tissues or even cells after treatment of the animal with the corresponding stimuli.

5.2.2. Comparison between pharmaceutical and genetic approaches

An additional cautionary note has to be made concerning data obtained with inhibitors. It is well known that many if not most pharmaceutical compounds have side effects. For some of them, off-target effects were minor or even negligible whereas for others, the off-target effects were even more pronounced then the specific on-target effects. For instance, concerning PARP-1 and Caspase-8 or 10, the novel types of PARP and Caspase inhibitors still inhibit the enzyme activity of all PARP or caspase family members [reviewed in ref. 4]. Finally, very recent reports indicate that certain

PARP as well as caspase inhibitors, possess free radical scavenging properties [159]. Since inflammation or stroke are both related to oxidative stress, it is quite possible that PARP or caspase inhibitors with antioxidative potency contribute indirectly and non-specifically to a decreased or increased NF-κB-dependent transcriptional activity by the reduction of free radicals. Thus, the exact role and contribution of the enzymatic activities of PARPs and caspases on NF-κB-dependent gene expression *in vivo* can only be addressed by profound genetic analyses using single or combinations of double and triple knock-in mice of enzymatic mutants of distinct PARP and caspase members.

5.3. Conclusions

A better understanding of the regulatory network modulating the interaction between NF-κB and PARP-1 or CARM-1 respectively, could provide new explanations for the aberrant T-cell development in CARM-1 deficient embryos and why PARP-1 -/- animals are protected against NF-κB regulated diseases such as septic shock, stroke and diabetes type 1. Deciphering the PARP-1 and CARM-1 dependent gene expression patterns, using genome wide approach, would provide us with the opportunities to develop new treatments to inhibit the undesired activity of NF-κB and thus, to block the inflammatory response in instances where this process became chronic. Indeed, the NF-κB PARP-1 or NF-κB CARM-1 interface might be obvious targets for new types of specific drugs which could interrupt specific protein-protein interactions and thus bearing much less side effects. Taken together, future investigation of the various roles of PARP-1 and CARM-1 in gene-specific regulation of transcription under normal physiological and pathophysiological conditions *in vivo*, would certainly represent an intense and exciting new field of research on its own.

6. Methods and materials

6.1. Cell culture

Primary mouse macrophage and lung fibroblast (MLF) cells were isolated from fresh littermates of 129S/EV-PARP-1 +/+ and 129S/EV-PARP-1 -/- mice according to an isolation procedure described in [135]. Primary mouse macrophages and the macrophage/monocyte-like murine Raw264 cells were grown in Hepes-buffered RPMI-Glutamax-I (Invitrogen) containing 10% FCS US/certified (Invitrogen) and supplemented with 50U/ml penicillin, 50µg/ml streptomycin (Invitrogen), 1mM sodium pyruvate, 0.05mM β-mercaptoethanol and MEM-NEAA. Primary mouse fibroblast cells were grown in Hepes-buffered DMEM-Glutamax-I (Invitrogen) containing 4.5g/L glucose, 10% FCS US/certified (Invitrogen) and supplemented with 50U/ml penicillin, 50µg/ml streptomycin (Invitrogen), 1mM sodium pyruvate, 0.05mM β-mercaptoethanol and MEM-NEAA. CARM-1 +/+ or CARM-1 –/– mouse embryonic fibroblasts (MEFs) were grown in Hepes-buffered DMEM-Glutamax-I (Invitrogen) containing 4.5g/L glucose and 10% FCS US/certified (Invitrogen) and supplemented with 50U/ml penicillin, 50µg/ml streptomycin (Invitrogen) and MEM-NEAA. Only cell passages 2 to 4 were used for all experiments.

6.1.1. Stimulation of the cells

For primary macrophages and macrophage/monocyte-like murine Raw264 cells, treatment with LPS was performed at a concentration of 0.1µg/ml where as for MLFs and MEFs a concentration of 1µg/ml LPS was used. Concerning the treatment with TNF-α, a concentration of 10ng/ml was used for all cell types. MEFs and MLFs were grown for 12 h in DMEM containing 2% fetal calf serum before stimulation with TNF-α or LPS. For some experiments cells were additionally pretreated with 50µM PJ-34, 20µM zVAD-fmk or 1% DMSO for 1h prior LPS treatment.

6.2. Preparation of RNA

RNA isolation from Primary mouse macrophage, mouse lung fibroblasts (MLFs) and mouse embryonic fibroblasts (MEFs) was performed using the Trizol procedure KIT from Invitrogen according to manufacturers protocols (Invitrogen).

6.3. RT-PCR

Reverse Transcription and PCR procedures were experimentally optimized based on original procedures from Promega (optimized parameters: length of random hexamer primers, annealing temperatures, annealing time and PCR elongation time for each gene specific primer pair, $MgCl_2$ concentration and number of Units/enzymes). The optimized procedures are shortly described below (see Table 4, 5, 6).

per RT reaction (total volume 50µl)
1. 33.2µl RNA (app. 300-500ng RNA in DEPC treated water)
2. denaturation at 70°C for 5 min
3. on ice for 10 min
4. 2µl of random hexamer primer (500µg/ml stock in DEPC treated water)
5. incubation at 70°C for 5 min
6. on ice
7. (no mastermix)
8. 10µl of 5x RT Buffer
9. 5µl of dNTP (10mM stock in DEPC treated water)
10. 0.3µl of 40U/µl RNasin (40U/µl stock)
11. 1.5µl of AMV Reverse Transcriptase (9U/µl stock)
12. incubation at 42°C for 60 min
13. on ice
14. inactivation at 95°C for 5 min
15. short centrifugation step
16. stored at -20°C.

Table 4. Optimized standard reverse transcription reaction procedure

per PCR-reaction (total volume 50µl)
1. 1µl cDNA (appr. 5-10ng)
2. 2µl of Fwd Primer (100µM stock)
3. 2µl of Rev Primer (100µM stock)
4. 44.6µl mastermix:
- 34.8µl of water
- 5µl of 10x Dynazyme Buffer with 15mM $MgCl_2$
- 3µl of 25mM $MgCl_2$ (final concentration 3mM)
- 1.8µl of 10mM dNTP Stock
5. 0.4µl Dynazyme (3U/µl stock)
6. PCR with optimum conditions for the certain primer (see table 6)

Table 5. Optimized standard PCR reaction procedure

gene	elongation time	annealing temperature
β-Actin	42 sec	50° C
COX-2	30 sec	55° C
GAPDH	45 sec	55° C
HPRT	60 sec	50° C
IκBα	50 sec	50° C
ICAM-1	45 sec	55° C
IL-6	50 sec	50° C
iNOS	60 sec	50° C
IP-10	45 sec	55° C
IRAK-4	60 sec	50° C
KC	45 sec	55° C
MCP-1	45 sec	55° C
M-CSF	45 sec	55° C
MIP-1α	60 sec	50° C
MIP-2	60 sec	50° C
PARP-1	60 sec	50° C
RANTES	60 sec	50° C
TLR-2	45 sec	50° C
TLR-4	45 sec	50° C
TNF-α	60 sec	50° C

Table 6. Optimized conditions for each gene-specific primer pair

6.3.1. Primer sequences

Gene	Length in bp	Forward-Primer	Review-Primer
β-Actin	569	5'-CAT GGA TGA CGA TAT CGC-3'	5'-CAT GAG GTA GTC TGT CAG G-3'
COX-2		5'-GCA AAT CCT TGC TGT TCC AAT C-3'	5'-GGA GAA GGC TTC CCA GCT TTT G-3'
GAPDH	983	5'-TGA AGG TCG GAG TCA ACG GAT TTG G-3'	5'-CAT GRG GGC CAT GAG GTC CAC CAC-3'
HPRT		5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3'	5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3'
IκBα		5'-GCC TTC CTC AAC TTC CAG AAC AAC-3'	5'-CAG ACG CTG GCC TCC AAA CAC ACA G-3'
ICAM-1	432	5'-TCG GAG GAT CAC AAA CGA AGC-3'	5'-AAC ATA AGA GGC TGC CAT CAC-3'
IL-6		5'-GAC AAA GCC AGA GTC CTT CAG AGA G-3'	5'-CTA GGT TTG CCG AGT AGA TCT C-3'
iNOS		5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3'	5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'
IP-10		5'-CCT ATC CTG CCC ACG TGT TGA G-3'	5'-CAC ACC TCC ACA TAG CTT ACA G-3'
IRAK-4		5'-GAT CCT CAA GAA GGG TGG AA-3'	5'-GCA AGC CCA AAG TCA GAT AT-3'
KC	530	5'-AAC GGA GAA AGA AGA CAG ACT GC-3'	5'-GAC GAG ACC AGG AGA AAC AGG G-3'
MCP-1		5'-TCT CTT CCT CCA CCA CCA TGC AG-3'	5'-GGA AAA ATG CAT CCA CAC CTT GC-3'
M-CSF	726	5'-AGT GAG GGA TTT TTG ACC CAG G-3'	5'-CTA TAC TGG CAG TTC CAC CTG TC-3'
MIP-1α	294	5'-AAC ATC ATG AAG GTC TCC AC-3'	5'-CCA AGA CTC TCA GGC ATT CA-3'
MIP-2	468	5'-ACT TCA GCC TAG CGC CAT GG-3'	5'-AGG TCA GTT AGC CTT GCC TT-3'
PARP-1		5'-GCA GAG TAT GCC AAG TCC AAC AG-3'	5'-ATC CAC CTC GTC GCC TTT TC-3'
RANTES	293	5'-GGT ACC ATG AAG ATC TCT GCA-3'	5'-AAA CCT TCT ATC CTA GCT CAT-3'
TLR-2	390	5'-CAG ACG TAG TGA GCG AGC TG-3'	5'-GGC ATC GGA TGA AAA GTG TT-3'
TLR-4	406	5'-CGA ATG TCT CTG GCA GGT GTA-3'	5'-CAA GGG ATA AGA ACG CTG AGA-3'
TNF-α		5'-ATG AGC ACA GAA AGC ATG ATC CGC-3'	5'-CCA AAG TAG ACC TGC CCG GAC TC-3'

Table 7. The sequences of each primer pair used in this study

6.3.2. Electrophoresis

All PCR products were resolved by 1-2% agarose gel electrophoresis and DNA visualized by staining with ethidium bromide according to Sambrook, Maniatis and Russell "Molecular Cloning (Third Edition). The electrophoresis running time was usually 30min (at 100V).

The length of the PCR products was controlled using a Lambda DNA/Pst I Marker. This marker contains DNA fragments with the length of 14057, 5077, 4749, 4507, 2838, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448 and 339bp.

6.4. List of material

Reagents for cell culture and cell stimulation

Recombinant TNF-α	R&D Systems
LPS (E. coli, O26:B6)	SIGMA
(prepared as dispersed sonicate in endotoxin-free water)	
PJ-34	Alexis
zVAD-fmk	SIGMA

Reverse Transcription

DEPC	SIGMA
Random Hexamer, pd$(N)_6$	Amersham Biosciences
RT Buffer	Promega
dNTP	Amersham Biosciences
AMV Reverse Transcriptase	Promega
RNasin Ribonuclease Inhibitor	Promega

PCR

PCR-tubes	Axygen
Dynazyme Buffer	Finnzyme
Primers	Microsynth
dNTP	Amersham Biosciences
$MgCl_2$	Finnzyme
Dynazyme	Finnzyme
PCR machine	AB Gene Amp PCR System 2700

Electrophoresis

Agarose	Eurobio
TAE, DNA Marker and loading buffer	prepared according to Sambrook, Maniatis and Russell “Molecular Cloning (Third Edition)
Ethidium bromide	SIGMA

7. References

1 May M.J. and Ghosh S. (1997) Rel/NF-kappa B and I kappa B proteins: an overview. Semin. Cancer Biol. **8:** 63-73

2 Pahl H.L., (1999) Activators and target genes of Rel/NF-κB transcription factors. Oncogene **18:** 6853-6866

3 Pahl H.L. (1999) Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene **18:** 6853-6866

4 Hassa P.O. and Hottiger M.O. (2002) The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-κB in inflammatory disorders. CMLS, Cell. Mol. Life Sci. **59:** 1534-1553

5 Rayet B., Gelinas C. (1999) Aberrant rel/nfkb genes and activity in human cancer. Oncogene **18:** 6938-6947

6 Brooks P.A., Nyborg J.K., Cockerell G.L. (1995) Identification of an NF-kappa B binding site in the bovine leukaemia virus promoter. J. Virol. **69:** 6005-6009

7 Flory E., Kunz M., Scheller C., Jassoy C., Stauber R., Rapp U.R., Ludwig S. (2000) Influenza virus-induced NF-kappaB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IkappaB kinase. J. Biol. Chem. **275:** 8307-8314

8 Oie K.L., Pickup D.J. (2001) Cowpox virus and other members of the orthopoxvirus genus interfere with the regulation of NF-kappaB activation. Virology **288:** 175-187

9 Kilk A., Talpsepp T., Vali U., Ustav M. (1996) Bovine papillomavirus oncoprotein E5 induces the NF kappa B activation through superoxide radicals. Biochem. Mol. Biol. Int. **40:** 689-697

10 Karin M., Ben-Neriah Y. (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev. Immunol. **18:** 621-663

11 Ghosh S., May M.J. and Kopp E.B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Re. Immunol. **16:** 225-260

12 Ryseck R-P., Bull P., Takamiya M., Bours V., Siebenlist U., Dobrzanski P., Bravo R. (1992) RelB, a new Rel family transcription activator that can interact with p50-NF-κB. Mol. Cell Biol. **12:** 674-684

13 Ryseck R-P., Novotny J., Bravo R. (1995) Characterization of elements determining the dimerization properties of RelB and p50. Mol. Cell Biol. **15:** 3100-3109

14 Bull P., Morley K.L., Hoekstra M.F., Hunter T., Verma I.M. (1990) The mouse c-rel protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. Mol. Cell Biol. **10:** 5473-5485

15 Schmitz M.L., Baeuerle P.A. (1991) The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10:** 3805-3817

16 Schmitz M.L., dos Santos Silva M.A., Altmann H., Szisch M., Holak T.A., Baeuerle P.A. (1994) Structural and functional analysis of the NF-κB p65 C terminus. An acidic and modular transactivation domain with the potential to adopt an alpha-helical conformation. J. Biol. Chem. **269:** 25613-25620

17 Blair W.S., Bogerd H.P., Madore S.J., Cullen B.R. (1994) Mutational analysis of the transcription activation domain of RelA: identification of a highly synergistic minimal acidic activation module. Mol. Cell Biol. **14:** 7226-7234

18 Schmitz M.L. Stelzer G., Altmann H., Meisterernst M., Baeuerle P.P. (1995) Interaction of the COOH-terminal transactivation domain of p65 NF-κB with TATA-binding protein, transcription factor IIB, and coactivators. J. Biol. Chem. **270:** 7219-7226

19 Dobrzanski P., Ryseck R-P., Bravo R. (1993) Both N- and C-terminal domains of RelB are required for full transactivation: role of the N-terminal leucine zipper-like motif. Mol. Cell Biol. **13:** 1572-1582
20 Kang S.M., Tran A.C., Grilli m., Lenardo M.J. (1992) NF-κB subunit regulation in nontransformed CD4+ T lymphocytes. Science **256:** 1452-1456
21 Lernbecher T., Muller U., Wirth T. (1993) Distinct NF-κB/Rel transcription factors are responsible for tissue-specific and inducible gene activation. Nature **365:** 767-770
22 Plaksin D., Baeuerle P.A., Eisenbach L. (1993) KBF1 (p50 NF-κB homodimer) acts as a repressor of H-2Kb gene expression in metastatic tumor cells. J. Exp. Med. **177:** 1651-1662
23 Brown A., Linhoff M., Stein B., Wright K., Baldwin A., Basta P., Ting J. (1994) Function of NF-κB/Rel binding sites in the major histocompatibility complex class II invariant chain promoter is dependent on cell-specific binding of different NF-κB/Rel subunits. Mol. Cell Biol. **14:** 2926-2935
24 Wulczyn F.G., Naumann M., Scheidereit C. (1992) Candidate proto-oncogene bdl-3 encodes a subunit-specific inhibitor of transcription factor NF-κB. Nature **358:** 597-599
25 Franzoso G., Bours V., Park S., Tomita Yamaguchi M., Kelly K., Siebenlist U. (1992) The candidate oncoprotein Bdl-3 is an antagonist of p50/ NF-κB-mediated inhibition. Nature **359:** 339-342
26 Franzoso G., Bours V., Azarenko V., Park S., Tomita-Yamaguchi M., Kanno T., Brown K., Siebenlist U. (1993) The oncoprotein Bcl-3 can facilitate NF-κB-mediated transactivation by removing inhibiting p50 homodimers from select κB sites. EMBO J. **12:** 3893-3901
27 Bours V., Franzoso G., Azarenko V., Park S., Kanno T., Brown K., Siebenlist U. (1993) The oncoprotein Bcl-3 directly transactivates through κB motifs via association with DNA-binding p50B homodimers. Cell **72:** 729-739
28 Fujita T., Nolan G.P., Liou H.C., Scott M.L., Baltimore D. (1993) The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-κB p50 homodimers. Genes Dev. **7:** 1354-1363
29 Nolan G.P., Fujita T., Bhatia K., Huppi C., Liou H.C., Scott M.L., Baltimore D. (1993) The bcl-3 proto-oncogene encodes a nuclear IκB-like molecule that preferentially interacts with NF-κB p50 and p52 in a phosphorylation-dependent manner. Mol. Cell Biol. **13:** 3557-3566
30 Rice N.R., MacKichan M.L., Israël A. (1992) The precursor of NF-κB p50 has IκB-like functions. Cell **71:** 243-253
31 Miyamoto S., Verma I.M. (1995) Rel/NF-kappa B/I kappa B story. Adv. Cancer Res. **66:** 255-292
32 Fan C.M., Maniatis T. (1991) Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature **345:** 395-398
33 Chen F.E., Huang D-B., Chen Y-Q., Ghosh G. (1998) Crystal structure of p50/p65 heterodimer of transcription factor NF-κB bound to DNA. Nature **391:** 410-413
34 Diehl J.A., Hannink M. (1994) Identification of a C/EBP-Rel complex in avian lymphoid cells. Mol. Cell Biol. **14:** 6635-6646
35 Ghosh G., van Duyne G., Ghosh S., Sigle P.B., (1995) Structure of NF-κB p50 homodimer bound to a κB site. Nature **373:** 303-310
36 Müler C.W., Harrison S.C. (1995) the structure of the NF-κB p50: DNA-complex: a starting point for analyzing the Rel family. FEBS Lett. **369:** 113-117
37 Karin M. (1998) The NF-kappa B activation pathway: its regulation and role in inflammation and cell survival. Cancer J. Schi. Am. **4 (suppl 1):** S92-S99
38 Beg A.A. and Baldwin A.J. Jr (1993) The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. Genes Dev. **7:** 2064-2070

39 Baeurle P.A., Baltimore D. (1998) IκB: a specific inhibitor of the NF-κB transcription factor. Science **242:** 540-546
40 Dobrzanski P., Ryseck R-P., Bravo R. (1995) Specific inhibition of ReIB/ p52 transcriptional activity by the C-terminal domain of p100. Oncogene **10:** 1003-1007
41 Jamaluddin M., Casola A., Garofalo R.P., Han Y., Elliott T. Ogra P.L., Brasier A.R. (1998) The major component of IκBα proteolysis occurs independently of the proteasome pathway in respiratory syncytial virus-infected pulmonary epithelial cells. J. Virol. **72:** 4849-4857
42 Wang Q., Dziarski R., Kirschning C.J., Muzio M., Gupta D. (2001) Micrococci and peptidoglycan activate TLR2→MyD88→IRAK→TRAF→NIK→IKK→NF-kappa signal transduction pathway that induces transcription of interleukin-8. Infect. Immun. **69:** 2270-2276
43 Palmer G.H., Machado J. Jr., Fernandez P., Heussler V., Perinat, Dobbelaere D.A. (1997) Parasite-mediated nuclear factor kappa B regulation in lymphoproliferation caused by Theileria parva infection. Proc. Natl. Acad. Sci. USA **11:** 12527-12532
44 Scheinman R.I., Cogswell P.C., Lofquist A.K., Baldwin A.S.Jr. (1995) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. Science **270:** 283-286
45 De Bosscher K., Vanden Berghe W., Vermeulen L., Plaisance S., Boone E., Haegeman G. (2000) Glucocoritcoids repress NF-kappaB-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. Proc. Natl. Acad. Sci. USA **97:** 3919-3924
46 Wang P., Wu P., Siegel M.I., Egan R.W., Billah M.M. (1995) Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monozytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J. Biol. Chem. **270:** 9558-9563
47 Pahl H.L., Krauss B., Schulze-Osthoff K., Decker T., Traenckner E.B., Vogt M. Myers C., Parks T., Warring P., Muhlbacher A., Czernilofsky A.P., Baeuerle P.A. (1996) The immunosuppressive fungal metabolite gliotoxin specially inhibits transcription factor NF-kappaB. J. Exp. Med. **183:** 1829-1840
48 Jin D.Y., Chae H.Z., Rhee S.G., Jeang K.T. (1997) Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. J. Biol. Chem. **272:** 30952-30961
49 Pierce J.W., Read M.A., Ding H., Luscinskas F.W., Collins T. (1996) Salicylates inhibit I kappa B-alpha phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration. J. Immunol. **156:** 3961-3969
50 Yin M.J., Yamamoto Y., Gaynor R.B. (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature **396:** 77-80
51 Meyer S., Kohler N.G., Joly A. (1997) Cyclosporine A is an uncompetitive inhibitor of proteasome activity and prevents NF-kappaB activation. FEBS Lett. **413:** 354-358
52 Tian B., Brasier A.R. (2003) Identification of a nuclear factor kappa B-dependent gene network. Recent Prog. Horm. Res. **58:** 95-130
53 May M.J. and Ghosh S. (1998) Signal transduction through NF-κB. Immunol. Today **19:** 80-88
54 Blobel G.A. (2000) CREB-binding protein and p300: molecular integrators of hematopoietic transcription. Blood **95:** 745-755
55 Covic M., Hassa P.O., Saccani S., Buerki C., Meier N.I., Lombardi C., Imhof R., Bedford M.T., Natoli G., Hottiger M.O. (2005) Arginine methyltransferase

promoter-specific regulator of NF-kappaB-dependent gene expression. EMBO J. **24:** 85-96
56 Hassa P.O., Buerki C., Lombardi C., Imhof R., Hottiger M.O. (2003) Transcriptional coactivation of nuclear factor-kappaB-dependent gene expression by p300 is regulated by poly(ADP)-ribose polymerase-1. J. Biol. Chem. **46:** 45145-45153
57 Hassa P.O. and Hottiger M.O. (1999) A Role of Poly(ADP-Ribose)Polymerase in NF-κB Transcriptional Activation. Biol Chem. **380(7-8):** 953-9
58 Shall S. (1983) ADP-ribosylation, DNA repair, cell differentiation and cancer. Princess Takamatsu Symp. **13:** 3-25
59 Lindahl T., Satoh M.S., Poirier G.G. and Klungland A. (1995) Post-translational modification of poly(ADP-ribose)polymerase induced by DANN strand breaks. Trends Biochem. Sci. **20:** 405-411
60 D'Amours D., Desnoyers S., D'Silva I. and Poirier G.G. (1999) Poly (ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem. J. **342:** 249-268
61 Alvarez-Gonzalez R., Pacheco-Rodriguez G. and Mendoza-Alvarez H. (1994) Enzymology of ADP-ribose polymer synthesis. Mol. Cell. Biochem. **138:** 33-37
62 Althaus F.R., Naegeli H., Realini C., Mathis G., Loetscher P. and Mattenberger M. (1990) The poly-ADP-ribosylation system of higher eukaryotes: a protein shuttle mechanism in chromatin? Acta Biol. Hung. **41:** 9-18
63 Althaus F.R. (1992) Poly-ADP-ribosylation: a histone shuttle mechanism in DNA excision repair. J. Cell Sci. **102:** 663-670
64 Kasid U.N., Halligan B., Liu L.F., Dritschilo A. and Smulson M. (1989) Poly(ADP-ribose)-mediated post-translational modification of chromatin-associated human topoisomerase I: inhibitory effects on catalytic activity. J. Biol. Chem. **264:** 18687-18692
65 Scovassi A.I., Mariani C., Neuroni M., Negri C. and Bertazzoni U. (1993) ADP-ribosylation of nonhistone proteins in HeLa cells: modification of DNA topoisomerase II. Exp. Cell Res. **206:** 177-181
66 Wesierska-Gadek J., Bugajska-Schretter A. and Cerni C. (1996) ADP-ribosylation of p53 tumor suppressor protein: mutant but not wild-type p53 is modified. J. Cell Biochem. **62:** 90-101
67 Boulikas T. (1991) Relation between carcinogenesis, chromatin structure and poly(ADP-ribosylation). Anticancer Res. **11:** 489-527
68 Kumari S.R., Mendoza-Alvarez H. and Alvarez-Gonzalez R. (1998) Functional interactions of p53 with poly(ADP-ribose)polymerase (PARP) during apoptosis following DNA damage: covalent poly(ADP-ribosyl)ation of p53 by exogenous PARP and noncovalent binding of p53 to the M(r) 85,000 proteolytic fragment. Cancer Res. **58:** 5075-5078
69 Bauer P.I., Buki K.G. and Kun E. (2001) Selective augmentation of histone H1 phosphorylation sites by interaction of poly(ADP-ribose)polymerase and cdc2-kinase: comparison with protein kinase C. Int. J. Mol. Med. **8:** 691-693
70 Bauer P.I., Chen H.J., Kenesi E., Kenessey I., Buki K.G., Kirsten E. et al. (2001) Molecular interactions between poly(ADP-ribose)polymerase (PARP I) and topoisomerase I (Topo I): identification of topology of binding. FEBS Lett. **506:** 239-242
71 Shall S. and Murcia G. de (2000) Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? Mutat. Res. **460:** 1-15
72 Le Rhun Y., Kirkland J.B. and Shah G.M. (1998) Cellular responses to DNA damage in the absence of poly(ADP-ribose) polymerase. Biochem. Biophys. Res. Commun. **245:**1-10
73 Gu Y., Sarnecki C., Aldape R.A., Livingston D.J. and Su M.S. (1995) Cleavage of poly(ADP-ribose) polymerase by interleukin-1 beta converting enzyme and its homologs TX and Nedd-2. J. Biol. Chem. **270:** 18715-18718

74 Tewari M., Quan L.T., O'Rourke K., Desnoyers S., Zeng Z., Beidler D.R. et al. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell **81:** 801-809
75 Amé J.C., Spenlehauer C., Murcia G. de (2004) The PARP superfamily. Bioessays. **26:** 882-893. Review.
76 Hassa P.O. et al. (2006) submitted
77 Smith S. (2001) The world according to PARP. Trends Biochem. Sci. **26:** 174-179
78 Ame J.C., Rolli V., Schreiber V., Niedergang C., Apiou F., Decker P. et al. (1999) PARP-2, a novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. J. Biol. Chem. **274:** 17860-17868
79 Johansson M. (1999) A human poly(ADP-ribose)polymerase gene family (ADPRTL): cDNA cloning of two novel poly(ADP-ribose)polymerase homologues. Genomics **57:** 442-445
80 Sallmann F.R., Vodenicharov M.D., Wang Z.Q. and Poirier G.G. (2000) Characterization of sPARP-1: an alternative product of PARP-1 gene with poly(ADP-ribose)polymerase activity independent of DNA strand breaks. J. Biol. Chem. **275:** 15504-15511
81 Smith S., Giriat I., Schmitt A. and Lange T. de (1998) Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. Science **282:** 1484-1487
82 Kaminker P.G., Kim S.H., Taylor R.D., Zebarjadian Y., Funk W.D., Morin G.B. et al. (2001) TANK2, a new TRF1-associated poly(ADP-ribose)polymerase, causes rapid induction of cell death upon overexpression. J. Biol. Chem. **276:** 35891-35899
83 Cook B.D., Dynek J.N., Chang W., Shostak G. and Smith S. (2002) Role for the related poly(ADP-ribose)polymerases tankyrase 1 and 2 at human telomeres. Mol. Cell Biol. **22:** 332-342
84 Murcia G. de, Schreiber V., Molinete M., Saulier B., Poch O., Masson M. et al. (1994) Structure and function of poly(ADP-ribose)polymerase. Mol. Cell Biochem. **138:** 15-24
85 Gradwohl G., Menissier de Murcia J.M., Molinete M., Simonin F., Koken M., Joeijmakers J.H. et al. (1990) The second zinc-finger domain of poly(ADP-ribose)polymerase determines specificity for single-stranded breaks in DNA. Proc. Natl. Acad. Sci. USA **87:** 2990-2994
86 Ikejima M., Noguchi S., Yamashita R., Ogura T., Sugimura T., Gill D.M. et al. (1990) The zinc fingers of human poly(ADP-ribose)polymerase are differentially required for the recognition of DNA breaks and nicks and the consequent enzyme activation: other structures recognize intact DNA. J. Biol. Chem. **265:** 21907-21913
87 Thibodeau J., Potvin F., Kirkland J.B. and Poirier G. (1993) Expression in Escherichia coli of the 36 kDa domain of poly(ADP-ribose)polymerase and investigation of its DNA binding properties. Biochim. Biophys. Acta **1163:** 49-53
88 Uchida K., Hanai S., Ishikawa K., Ozawa Y., Uchida M., Sugimura T. et al. (1993) Clonino of cDNA encoding Drosophila poly(ADP-ribose)polymerase: leucine zipper in the automodification domain. Proc. Natl. Acad. Sci. USA **90:** 3481-3485
89 Busch S.J. and Sassone-Corsi P. (1990) Dimers, leucine zippers and DNA-binding domains. Trends Genet. **6:** 36-40
90 Callebaut I. and Mornon J.P. (1997) From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. FEBS Lett. **400:** 25-30
91 Kim J.W., Kim K., Kang K. and Joe C.O. (2000) Inhibition of homodimerization of poly(ADP-ribose)polymerase by its C-terminal cleavage products produced during apoptosis. J. Biol. Chem. **275:** 8121-8125

92 Kawaichi M., Oka J., Zhang J., Ueda K. and Hayaishi O. (1983) Properties of poly(ADP-ribose) synthetase and ADP-ribosyl histone splitting enzyme. Princess Takamatsu Symp. **13:** 121-128
93 Duriez P.J., Desnoyers S., Hoflack J.C., Shah G.M., Morelle B., Bourassa S. et al. (1997) Characterization of anti-peptide antibodies directed towards the automodification domain and apoptotic fragment of poly(ADP-ribose)polymerase. Biochim. Biophys. Acta. **1334:** 65-72
94 Mendoza-Alvarez H. and Alvarez-Gonzalez R. (1999) Biochemical characterization of mono(ADP-ribosyl)ated poly(ADP-ribose)polymerase. Biochemistry **38:** 3948-3953
95 Trucco C., Rolli V., Oliver F.J., Flatter E., Masson M., Dantzer F. et al. (1999) A dual approach in the study of poly(ADP-ribose)polymerase: in vitro random mutagenesis and generation of deficient mice. Mol. Cell. Biochem. **193:** 53-60
96 Jung S., Miranda E.A., Murcia J.M. de, Niedergang C., Delarue M., Schulz G.E. et al. (1994) Crystallization and X-ray crystallographic analysis of recombinant chicken poly(ADP-ribose)polymerase catalytic domain produced in Sf9 insect cells. J. Moll. Biol. **244:** 114-116
97 Ogura T., Takenouchi N., Yamaguchi M., Matsukage A., Sugimura T. and Esumi H. (1990) Striking similarity of the distribution patterns of the poly(ADP-ribose)polymerase and DNA polymerase beta among various mouse organs. Biochem. Biophys. Res. Commun. **172:** 377-384
98 Menegazzi M., Grassi-Zucconi G., Carcerero De Prati A., Ogura T., Poltronieri P., Nyunoya H. et al. (1991) Differential expression of poly(ADP-ribose)polymerase and DNA polymerase beta in rat tissues. Exp. Cell Res. **197:** 66-74
99 Dantzer F., La Rubia G. de, Menissier-de Murcia J., Hostomsky Z., Murcia G. de and Schreiber V. (2000) Base excision repair is impaired in mammalian cells lacking poly(ADP-ribose)polymerase-1. Biochemistry **39:** 7559-7569
100 Zucconi G.G., Carcereri de Prati A., Menegazzi M., Cosi C. and Suzuki H. (1992) DNA repair enzymes in the brain: DNA polymerase beta and poly(ADP-ribose)polymerase. Ann. NY Acad. Sci. **663:** 432-435
101 Menegazzi M., Gerosa F., Tommasi M., Uccida K., Miwa M., Sugimura T. et al. (1998) Induction of poly(ADP-ribose)polymerase gene expression in lectin-stimulated human T lymphocytes is dependent on protein synthesis. Biochem. Biophys. Res. Commun. **156:** 995-999
102 McNerney R., Tavasolli M., Shall S., Brazinski A. and Johnstone A. (1989) Changes in mRNA levels of poly(ADP-ribose)polymerase during activation of human lymphocytes. Biochim. Biophys. Acta. **1009:** 185-187
103 Concha I.I., Figueroa J., Choncha M.I., Ueda K. and Burzio L.O. (1989) Intracellular distribution of poly(ADP-ribose)synthetase in rat spermatogenic cells. Exp. Cell Res. **180:** 353-366
104 Kanai M., Uchida M., Hanai S., Uematsu N., Uchida K. and Miwa M. (2000) Poly(ADP-ribose)polymerase localizes to the centrosomes and chromosomes. Biochem. Biophys. Res. Commun. **278:** 385-389
105 Desnoyers S., Kirkland J.B. and Poirier G.G. (1996) Association of poly(ADP-ribose)polymerase with nuclear subfractions catalyzed with sodium tetrathionate and hydrogene peroxide crosslinks. Mol. Cell Biochem. **159:** 155-161
106 Ha H.C., Hester L.D., Snyder S.H. (2002) Poly(ADP-ribose)polymerase-1 dependence of stress-induced transcription factors and associated gene expression in glia. Proc. Natl. acad. Sci. USA. **99(5):** 3270-3275
107 Hassa P.O., Covic M., Hasan S., Imhof R., Hottiger M.O. (2001) The enzymatic and DNA binding activity of PARP-1 are not required for NF-kappa B coactivator function. J. Biol. Chem. **276(49):** 45588-45597

108 Liaudet L., Pacher P., Mabley J.G., Virag L., Soriano F.G., Hasko G. et al. (2002) Activation of poly(ADP-ribose) polymerase-1 is a cntral mechanism of lipopolysaccharide-induced acute lung inflammation. Am. J. Respir. Crit. Care Med. **165:** 372-377
109 Soriano F.G., Pacher P., Mabley J., Liaudet L., Szabo C. (2001) Rapid reversal of the diabetic endothelial dysfunction by pharmacological inhibition of poly(ADP-ribose)polymerase. Circ. Res. **89(8):** 684-691
110 Szabo C., Lim. L.H., Cuzzocrea S. et al. (1997) Inhibition of poly(ADP-ribose) synthetase attenuates neutrophil recruitment and exerts antiinflammatory effects. J. Exp. Med. **186(7):** 1041-1049
111 Cuzzocrea S., Chatterjee P.K., Mazzon E., Dugo L., Serraino I., Britti D. et al. (2002) Pyrrolidine dithicarbamate attenuates the development of acute and chronic inflammation. Br. J. Pharmacol. **135:** 496-510
112 Soriano F.G., Virag L. and Szabo C. (2001) Diabetic endothelial dysfunction role of reactive oxygen and nitrogen species production and poly(ADP-ribose) polymerase activation. J. Mol. Med. **79:** 437-448
113 Cuzzocrea S., McDonald M.C., Mazzon E., et al. (2002) Effects of 5-aminoisoquinolinone, a water-soluble, potent inhibitor of the activity of poly(ADP-ribose)polymerase, in a rodent model of lung injury. Biochem. Pharmacol. **63(2):** 293-304
114 Tentori L., Portarena I., Graziani G. (2002) Potential clinical applications of poly(ADP-ribose)polymerase (PARP) inhibitors. Pharmacol. Res. **45(2):** 73-85
115 Ha H.C. and Snyder S.H. (2000) Poly(ADP-ribose) polymerase-1 in the nervous system. Neurobiol. Dis. **7:** 225-239
116 Rossol M., Gartner D., Hauschildt S. (2001) Diverse regulation of microfilament assembly, production of TNF-alpha, and reactive oxygen intermediates by actin modulating substances and inhibitors of ADP-ribosylation in human monocytes stimulated with LPS. Cell. Motil Cytoskeleton. **48(2):** 96-108
117 Hauschildt S., Scheipers P., Bessler W., et al. (1997) Role of ADP-ribosylation in activated monocytes/macrophages. Adv. Exp. Med. Biol. **419:** 249-252
118 Koch-Nolte F., Haag F. (1997) Mono(ADP-ribosyl)transferases and related enzymes in animal tissues. Emerging gene families. Adv. Exp. Med. Biol. **419:**1-13
119 Pieper A.A., Blackshaw S., Clements E.E., et al. (2000) Poly(ADP-ribosyl)ation basally activated by DNA strand breaks reflects glutamate-nitritic oxide neurotransmission. Proc. Natl. Acad. Sci. USA. **97(4):** 1845-1850
120 Veres B., Gallyas F. Jr., Varbiro G., et al. (2003) Decrease of the inflammatory response and induction of the Akt/protein kinase B pathway by poly(ADP-ribose)polymerase 1 inhibitor in endotoxin-induced septic shock. Biochem. Pharmacol. **65(8):** 1373-1382
121 Chu W., Gong X., Li Z., et al. (2000) DNA-Pkcs is required for activation of innate immunity by immunostimulatory DNA. Cell. **103(6):** 909-918
122 Plevy S.E., Gemberling J.H., Hsu S., Dorner A.J., Smale S.T. (1997) Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. Mol. Cell. Biol. **17(8):** 4572-4588
123 Catron K.M., Brickwood J.R., Shang C., Li Y., Shannon M.F., Parks T.P. (1998) Cooperative binding and synergistic activation by RelA and C/EBPbeta on the intercellular adhesion molecule-1 promoter. Cell Growth Differ. **9(11):** 949-959
124 Sakitani K., Nishizawa M., Inoue K., Masu Y., Okumura T., Ito S. (1998) Synergistic regulation of inducible nitric oxide synthase gene by CCAAT/enhancer-binding protein beta and nuclear factor-kappaB in hepatocytes. Genes Cells. **3(5):** 321-330

125 Roebuck K.A., Rahman A., Lakshminarayanan V., Janakidevi K., Malik A.B. (1995) H2O2 and tumor necrosis factor-elements within the ICAM-1 promoter. J. Biol. Chem. **270(32):** 18966-18974
126 Czapski G.A., Cakala M., Kopeczuk D., Strosznajder J.B. (2004) Effect of poly(ADP-ribose)polymerase inhibitors on oxidative stress evoked hydroxyl radical level and macromolecules oxidation in cell free system of rat brain cortex. Neurosci Lett. **356(1):** 45-48
127 Szabo C. (1998) Role of poly(ADP-ribose)synthetase in inflammation. Eur J Pharmacol. **350(1):** 1-19
128 Szabo C., Dawson V.L. (1998) Role of poly(ADP-ribose)synthetase in inflammation and ischaemia-reperfusion. Trends Pharmacol. Sci. **19(7):** 287-298
129 Halmosi R., Berente Z., Osz E., Toth K., Literati-Nagy P. and Sumegi B. (2001) Effect of poly(ADP-ribose) polymerase inhibitors on the ischemia-reperfusion-induced oxidative cell damage and mitochondrial metabolism in Langendorff heart perfusion system. Mol. Pharmacol. **59:** 1497-1505
130 Abdelkarim G.E., Gertz K., Harms C., Katchanov J., Dirnagl U., Szabo C. et al. (2001) Protective effects of PJ34, a novel, potent inhibitor of poly(ADP-ribose)polymerase (PARP) in in vitro and in vivo models of stroke. Int. J. Mol. Med. **7:** 255-260
131 Mabley J.G., Jagtap P., Perretti M., Getting S.J., Salzmann A.L., Virag L. et al. (2001) Anti-inflammatory effects of a novel potent inhibitor of poly(ADP-ribose) polymerase. Inflamm. Res. **50:** 561-569
132 Ullrich O., Diestel A., Eyupoglu I.Y., Nitsch R. (2001) Regulation of microglial expression of integrins by poly(ADP-ribose)polymerase-1. Nat Cell Biol. **3(12):** 1035-1042
133 Hassa P.O., Haenni S.S., Buerki C., Meier N.I., Lane W.S., Gersbach M., Imhof R., Hottiger M.O. (2005) Acetylation of poly(ADP-ribose)polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription. J. Biol. Chem. **280(49):** 40450-40464
134 Mabley J.G., Suarez-Pinzon W.L., Hasko G., Salzman A.L., Rabinovitch A., Kun E. et al. (2001) Inhibition of poly(ADP-ribose) synthetase by gene disruption or inhibition with 5-iodo-6-amino-1,2-benzopyrone protects mice from multiple-low-dose-streptozotocin-induced diabetes. Br. J. Pharmacol. **133:** 909-919
135 Pétrilli V., Herceg Z., Hassa P.O., Wang Z-Q et al. (2004) Noncleavable poly(ADP-ribose) polymerase-1 regulates the inflammation response in mice. Clin. Invest. **114:** 1072-1081
136 Eliasson M.J., Sampei K., Mandir A.S., Hurn P.D., Traystman R.J., Bao J. et al. (1997) Poly(ADP-ribose)polymerase gene disruption renders mice resistant to cerebral ischemia. Nat. Med. **3:** 1089-1095
137 Heller B., Wang Z.Q., Wagner E.F., Radons J., Burkle A., Fehsel K. et al. (1995) Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. J. Biol. Chem. **270:** 11176-11180
138 Virag L., Scott G.S., Cuzzocrea S., Marmer D., Salzmann A.L. and Szabo C. (1998) Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly(ADP-ribose) synthetase (PARS) activation. Immunology **94:** 345-355
139 Ha H.C. and Snyder S.H. (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc. Natl. Acad. Sci. USA **96:** 13978-13982
140 Burkart V., Wang Z.Q., Radons J., Heller B., Herceg Z., Stingl L. et al. (1999) Mice lacking the poly(ADP-ribose) polymerase gene are resistent to pancreatic beta-cell destruction and diabetes development induced by streptozotocin. Nat. Med. **5:** 314-319

141 Pieper A.A., Verma A., Zhang J. And Snyder S.H. (1999) Poly(ADP-ribose)polymerase, nitric oxide and cell death. Trends Pharmacol. Sci. **20:** 171-181
142 Wang Z.Q., Stingl L., Morrison C., Jantsch M., Los M., Schulze-Osthoff K. et al. (1997) PARP is important for genomic stability but dispensable in apoptosis. Genes Dev. **11:** 2347-2358
143 Trucco C., Oliver F.J., Murcia G. de and Menissier-de Murcia J. (1998) DNA repair defect in poly(ADP-ribose)polymerase-deficient cell lines. Nucleic Acids Res. **26:** 2644-2649
144 Masutani M., Nozaki T., Nishiyama E., Shimokawa T., Tachi Y., Suzuki H. et al. (1999) Function of poly(ADP-ribose)polymerase in response to DNA damage: gene disruption study in mice. Mol. Cell. Biochem. **193:** 149-152
145 Masutani M., Nozaki T., Watanabe M., Ochiya T., Hasegawa F., Nakagama H. et al. (2001) Involvement of poly(ADP-ribose) polymerase in trophoblastic cell differentiation during tumorigenesis. Mutat Res. **477:** 111-117
146 Tsutsumi M., Masutani M., Nozaki T., Kusuoka O., Tsujiuchi T., Nakagama H. et al. (2001) Increased susceptibility of poly(ADP-ribose) polymerase 1 knockout mice to nitrosamine carcinogenicity. Carcinogenesis **22:** 1-3
147 Tong W.M., Hande M.P., Lansdorp P.M. and Wang Z.Q. (2001) DNA strand break-sensing molecule poly(ADP-ribose) polymerase cooperates with p53 in telomere function, chromosome stability, and tumor suppression. Mol. Cell Biol. **21:** 4046-4054
148 Conde C., Mark m., Oliver F.J., Huber A., Murcia G. de and Menissier-de Murcia J. (2001) Loss of poly(ADP-ribose) polymerase-1 causes increased tumour latency in p53-deficient mice. EMBO J. **20:** 3535-3543
149 Simbulan-Rosenthal C.M., Ly D.H. Rosenthal D.S., Konopka G., Luo R., Wang Z.Q. et al. (2000) Misregulaion of gene expression in primary fibroblasts lacking poly(ADP-ribose) polymerase. Proc. Natl. Acad. Sci. USA **97:** 11274-11279
150 Pieper A.A., Brat D.J., Krug D.K., Watkins C.C., Gupta A., Blackshaw S. et al. (1999) Poly(ADP-ribose)polymerase-deficient mice are protected from streptozotocin-induced diabetes. Proc.Natl. Acad. Sci. USA **96:** 3059-3064
151 Szabo C., Lim L.H., Cuzzocrea S., Getting S.J., Zingarelli B., Flower R.J. et al. (1997) Inhibition of poly(ADP-ribose)synthetase attenuates neutrophil recruitment and exerts anti-inflammatory effects. J. Exp. Med. **186:**1041-1049
152 Oliver F.J., Menissier-de Murcia J., Nacci C., Decker P., Andriantsitohaina R., Muller S. et al. (1999) Resistance to endotoxic shock as a consequence of detective NF-kappaB activation in poly(ADP-ribose)polymerase-1 deficient mice. EMBO J. **18:** 4446-4454
153 Kuhnle S., Nicotera P., Wendel A. and Leist M. (1999) Prevention of endotoxin-induced lethality, but not of liver apoptosis in poly(ADP-ribose) polymerase-deficient mice. Biochem. Biophys. Res. Commun. **263:** 433-438
154 Brownlee M. (2001) Biochemistry and molecular cell biology of diabetic complications. Nature **414:** 813-820
155 Mathis D., Vence L. and Benoist C. (2001) Beta-cell death during progression to diabetes. Nature **414:** 792-798
156 Roden M.M., Brims D.R., Fedorov A.A., Dolorenzo T.P., Almo S.C., Nathenson S.G. (2005) Structural analysis of H2-D(b) class I molecules containing two different allelic forms of the type 1 diabetes susceptibility factor beta-2 microglobulin: Implications for the mechanism underlying variations in antigen presentation. Mol Immunol. (Epub ahead of print)
157 Like A.A. and Rossini A.A. (1976) Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. Science **193:** 415-417
158 Rossini A.A., Like A.A., Chick W.L., Appel M.C. and Cahill G.F.Jr (1977) Studies of streptozotocin-induced insulitis and diabetes. Proc. Natl. Acad. Sci. USA **74:** 2485-2489

159 Schnedl W.J., Ferber S., Johnson J.H. and Newgard C.B. (1994) STZ transport and cytotoxicity: specific enhancement in GLUT2-expressing cells. Diabetes **43:** 1326-1333
160 Soriano F.G., Pacher P., Mabley J., Liaudet L. and Szabo C. (2001) Rapid reversal of the diabetic endothelial dysfunction by pharmacological inhibition of poly(ADP-ribose) polymerase. Circ. Res. **89:** 684-691
161 Mabley J.G., Pacher P., Southan G.J., Salzman A.L. and Szabo C. (2002) Nicotine reduces the incidence of type I diabetes in mice. J. Pahrmacol. Exp. Ther. **300:** 876-881
162 Sandler S., Bendtzen K., Eizirik D.L., Strandell E., Welsh M. and Welsh N. (1990) Metabolism and beta-cell function of rat pancreatic islets exposed to human interleukin-1 beta in the presence of a high glucose concentration. Immunol. Lett. **26:** 245-251
163 Rabinovitch A. (1992) Free radicals as mediators of pancreatic islet beta-cell injury in autoimmune diabetes. J. Lab. Clin. Med. **119:** 455-456
164 Schmidt A.M., Hori O., Cao R., Yan S.D., Brett J., Wautier J.L. et al. (1996) RAGE: a novel cellular receptor for advanced glycation end products. Diabetes **45 (suppl. 3):** S77-S80
165 Yeh C.H., Sturgis L., Haidacher J., Zhang X.N., Sherwood S.J., Bjercke R.J. et al. (2001) Requirement for p38 and p44/p42 mitogen-activated protein kinases in RAGE-mediated nuclear factor-kappaB transcriptional activation and cytokine secretion. Diabetes **50:** 1495-1504
166 Garcia Soriano F., Virag L. Jagtap P., Szabo E., Mabley J.G., Liaudet L. et al. (2001) Diabetic endothelial dysfunction: the role of poly(ADP-ribose) polymerase activation. Nat. Med. **7:** 108-113
167 Masutani M., Suzuki H., Kamada N., Watanabe M., Ueda O., Nozaki T. et al. (1999) Poly(ADP-ribose) polymerase gene disruption conferred mice resistant to streptozotocin-induced diabetes. Proc. Natl. Acad. Sci. USA **96:** 2301-2304
168 Downey J.S. and Han J. (1998) Cellular activation mechanisms in septic shock. Front. Biosci. **3:** 468-476
169 Zingarelli B., Day B.J., Crapo J.D., Salzman A.L. and Szabo C. (1997) The potential role of peroxynitrite in the vascular contractile and cellular energetic failure in endotoxic shock. Br. J. Pharmacol. **120:** 259-267
170 Szabo C. Cuzzocrea S., Zingarelli B., O'Connor M. and Salzman A.L. (1997) Endothelial dysfunction in a rat model of endotoxic shock: importance of the activation of poly(ADP-ribose) synthetase by peroxynitrite. J. Clin. Invest. **100:** 723-735
171 Wray G.M., hinds C.J. and Thiemermann C. (1998) Effects of inhibitors of poly(ADP-ribose) synthetase activity on hypotension and multiple organ dysfunction caused by endotoxin. Shock **10:** 13-19
172 Saccani S., Pantano S., Natoli G. (2001) Two waves of nuclear factor kB recruitment to target promoters. J. Exp. Med. **193:** 1351-1359
173 de Kok J.B., Roelofs R.W., Giesendorf B.A., Pennings J.L., Waas E.T., Feuth T., Swinkels D.W., Span P.N. (2005) Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab. Invest. **85(1)**:154-159
174 www.copewithcytokines.de/cope.cgi
175 Heyninck K. and Beyaert R. (2001) Crosstalk between NF-kappaB-activating and apoptosis-inducing proteins of the TNF-receptor complex. Mol Cell Biol Res Commun. **4(5)**:259-65. Review.
176 Chaudhary PM et al. (2000) Activation of the NF-kappaB pathway by caspase 8 and its homologs. Oncogene **19(39)**: 4451-60.
177 Shikama Y et al. (2003) Caspase-8 and caspase-10 activate NF-kappaB through RIP, NIK and IKKalpha kinases. Eur J Immunol. **33(7):** 1998-2006

178 Hassa P.O., Hottiger M.O. (2005) An epigenetic code for DNA damage repair pathways? Biochem Cell Biol. **83:** 270-285 Review.
179 Dohrman A., Russell J.Q., Cuenin S., Fortner K., Tschopp J., Budd R.C. (2005) Cellular FLIP long form augments caspase activity and death of T cells through heterodimerization with and activation of caspase-8. J Immunol. **175:** 311-318
180 Wang H., Li J., Follett P.L., Zhang Y., Cotanche D.A., Jensen F.E., Volpe J.J., Rosenberg P.A. (2004) 12-Lipoxygenase plays a key role in cell death caused by glutathione depletion and arachidonic acid in rat oligodendroctes.Eur J Neurosci. **20:** 2049-2058

8. Annex A

Gene	*Function*
Cytokines/Chemokines and their modulators	
CCL5	T-cell secreted factor
CCL15/Leukotactin	Chemokine for cell attraction
CCL22	Epithelial cell chemokine at mucosa
CCL28	Chemokine for T-cell attraction
CINC-1	Cytokine-induced neutrophil chemoattractant
*CXCL 11	Chemokine ligand for CXCR3
Eotaxin	β-Chemokine, eosinophil-specific
Fractalkine	TNF-induced chemokine
Gro α-γ	Melanoma growth stimulating activity
Gro-1	Growth regulated oncogene; chemokine
*ICOS	Inducible co-stimulator
IFN-γ	Interferon
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-1 receptor antagonist	Inhibitor of IL-1 activity
IL-2	Interleukin-2
IL-6	Interleukin-6, inflammatory cytokine
IL-8	Interleukin-8, α-chemokine
*IL-9	Interleukin-9
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-12 (p40)	Interleukin-12
IL-13	Interleukin-13
*IL-15	Interleukin-15
β-Interferon	Interferon
IP-10	α chemokine
KC	α chemokine
*LIX (mouse); ENA-78 (CXCL5) and GCP-2 (CXCL6) (human)	LPS-induced CXC chemokines
Lymphotoxin α	TNF-like cytokine
Lymphotoxin β	Anchors TNF to cell surface
MCP-1/JE	Macrophage chemotactic protein, β chemokine
MIP-1α, β aka (LAG-1)	Macrophage inflammatory protein-1, β chemokine Lymphocyte activation gene-1
MIP-2	Macrophage inflammatory protein-2, β chemokine
MIP-3α	Macrophage inflammatory protein-3α
*mob-1	A-C-X-C chemokine
Neutrophil activating peptide-78	Activates neutrophils
RANTES	Regulated upon activation of normal T lymphocyte expressed and secreted, β chemokine
TCA3, T-cell activation gene 3	T-cell activation gene 3, β chemokine
TNFα	Tumor necrosis factor α
TNFβ	Tumor necrosis factor β
TRAIL (aka Apo2 ligand)	Cytokine
TFF3 (Treefoil factor)	Peptide in response to gut irritation
VEGI	Vascular endothelial growth inhibitor
Immunoreceptors	
B7.1 (CD80)	Co-stimulation of T cells via CD28 binding
BRL-1	B-cell homing receptor
CCR5	Chemokine receptor
CCR7	Chemokine receptor
CD137	TNF-like receptor
CD154	CD40 ligand

CD40	TNF-receptor family member
CD48	Antigen of stimulated lymphocytes
CD83	T-cell development molecule
Fc ε receptor II (CD23)	Receptor for IgE
IL-2 receptor α-chain	IL-2 receptor subunit
Immunoglobulin Cγ1	IgG heavy chain
Immunoglobulin γ4	IgG heavy chain
Immunoglobulin ε heavy chain	IgE heavy chain
Immunoglobulin κ light chain	Antibody light chain
Invariant Chain I_l	Antigen presentation
MHC class I (H-2Kb)	Major histocompatibility antigen
MHC class I HLA-B7	Major histocompatibility antigen
β2 Microglobulin	Chaperone for MHC class I-like molecules
Nod2	Intracellular pathogen recognition
Polymeric Ig receptor	Binds Ig
T-cell receptor β chain	T-cell receptor subunit
T-cell receptor/CD3γ	T-cell receptor subunit
TLR-2	Toll-like receptor
TLR-9	Toll-like receptor
*TNF-Receptor, p75/80 (CD120B)	High-affinity TNF receptor
Proteins involved in antigen presentation	
Complement B	Activator of alternative complement pathway
Complement component 3	Component of complement pathway
Complement Receptor 2	Complement receptor for B-cell response to Ag
Proteasome Subunit LMP2	Subunit of 26S proteasome, cysteine protease
Peptide Transporter TAP1	Peptide transporter for ER
Tapasin	MHC class I presentation and assembly
Cell adhesion molecules	
DC-SIGN	Dendritic cell surface C-type lectin
ELAM-1 (CD62E, E-selectin)	E-selectin, endothelial cell leukocyte adhesion molecule
Endoglin	Endothelial cell membrane glycoprotein
Fibronectin	Extracellular attachment
ICAM-1	Intercellular adhesion molecule-1
Mad CAM-1	Mucosal addressin cell adhesion molecule
P-select6in	Platelet adhesion receptor
Tenascin-C	ECM protein controls cell attachment and migration, cell growth
VCAM-1	Vascular cell adhesion molecule
Acute phase proteins	
Angiotensinogen	Angiotensin precursor, regulates blood pressure
β-defensin-2	Anti-microbial peptide
C4b binding protein	Complement binding protein
Complement factor B	Complement factor
Complement factor C4	Activates extrinsic pathway of complement activation
C-reactive protein	Host defense protein
Lipopolysaccharide binding protein	Binds to LPS receptor (CD14) with LPS
Pentraxin PTX3	Pentraxin
Serum amyloid a proteins (SAA1, SAA2, SAA3)	Serum components
Tissue factor-1	Activates extrinsic pathway of complement activation
Urokinase-type plaminogen activator	Activates fibrinogen for fibrin clot lysis
Stress response genes	
Angiotensin II	Peptide hormone
*CYP2E1	Cytochrome p450
CYP2C11	Cytochrome p450
COX-2	Cyclooxygenase, prostaglandin endoperoxide synthase

Ferritin H	Iron storage protein
*5-Lipoxygenase (guinea pig)	Arachidonic acid metabolic enzyme, leukotriene synthesis
12-Lipoxygenase	Arachidonic acid metabolic enzyme
Inducible NO-Synthase	NO synthesis
*MAP4K1	Activator of stress-induced protein kinase pathway
Cu/Zn SOD	Superoxide dismutase
Mn SOD	Superoxide dismutase
NAD(P)H quinine oxidoreductase (DT-diaphorase)	Bioreductive enzyme
Phospholipase A2	Fatty acid metabolism
Cell-surface receptors	
A1 adenosine receptor	Pleiotropic physiological effects
Amiloride-sensitive sodium channel	Sodium channel
*α2B-adrenergic receptor	Adrenergic receptor
Bradykinin B1-Receptor	Pleiotropic physiological effects
*CD23	Cell-surface molecule
CD69	Lectin mainly on activated T-cells
*Epidermal Growth Factor Receptor	Receptor for EGF
Gal1 Receptor	Galanin receptor neuroendocrine peptide
Lox-1	Receptor for Oxidized low density lipoprotein
Mdr1	Multiple drug resistance mediator (P-glycoprotein)
μ-opioid receptor	Opioid Receptor
Neuropeptide Y-Y1 receptor	Pleiotropic physiological effects
*NMDA receptor subunit 2A (rat)	Neural receptor for N-methyl-D-aspartate
*NMDA receptor subunit NR-1 (GRIN1 gene)	Subunit of neural receptor for N-methyl-D-aspartate
PAF receptor 1	Platelet activator receptor
RAGE-receptor for advanced glycation end products	Receptor for advanced glycation end products
Regulators of apoptosis	
Bax	Pro-apoptotic Bcl-2 homologue
Bfl1/A1	Pro-survival Bcl-2 homologue
Bcl-xL	Pro-survival Bcl-2 homologue
Bcl-2	Pro-survival factor
Caspase-11	Caspase
Nr13	Pro-survival Bcl-2 homologue
c-FLIP	Pro-survival factor
CD95 (Fas)	Pro-apoptotic receptor
*Fas-associated phosphatase-1	Protein phosphatase
Fas-Ligand	Inducer of apoptosis
IAPs	Inhibitors of apoptosis
IEX-1L	Immediate early gene
TRAF-1	TNF-receptor associated factor
TRAF-2	TNF-receptor associated factor
Growth factors, ligands, and their modulators	
Angiopoietin	Tie-2 receptor ligand
BMP-2	Bone morphogenic protein-2
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
*HGF/SF	Hepatocyte growth factor/scatter factor
EPO	Erythropoietin
*IGFBP-1	Insulin-like growth factor binding protein-1
M-CSF (CSF-1)	Insulin-like growth factor binding protein-2
NK-1R	Macrophage colony stimulating factor
NK4	Neurokinin-1 receptor
PDGF B chain	Hepatocyte growth factor
Proenkephalin	Hormone
Stem cell factor	Mast cell growth factor

*Thrombospondin-1 (TSP-1)	Matrix glycoprotein t
*Thrombospondin-2 (THBS2)	Matrix glycoprotein t
VEGF C	Vascular endothelial growth factor
Early response genes	
*B94	Early response gene
*Egr-1	Mitogen-induced early response gene; zinc finger
p22/PRG1	Rat homologue of IEX
*p62	Non-proteasomal multi-ubiquitin chain binding protein
*TIEG	TGF-β early response gene; zinc finger protein
Transcription factors	
A20	TNF-inducible zinc finger
Androgen receptor	Hormone receptor
*c-fos (fish gene)	Proto-oncogene
c-myb	Proto-oncogene
c-myc	Proto-oncogene
c-rel	Proto-oncogene
E2F3a	Cell cycle regulator
Elf3	Ets family transcription factor
*ELYS	Embryonic large molecule derived from yolk sac
*ETR101	TPA-inducible, Jun-like transcription factor
*Glucocorticoid receptor	Promotor 1B of the GR
IRF-1	Interferon regulatory factor-1
IRF-2	Interferon regulatory factor-2
IRF-4	Interferon regulatory factor-4
IRF-7	Interferon regulatory factor-7
IκB-α	Inhibitor of Rel/NF-κB
junB	Proto-oncogene
Mail	IκB-like protein
NF-κB2	NF-κB p100 precursor
NF-κB1	NF-κB p105 precursor
NURR1	Nuclear orphan receptor
P53	TF, Tumor suppressor
Relb	Transcription factor
Snail	Transcription factor
Sox9	Transcription factor
Stat5a	Transcription factor
WT1	Zinc finger transcription factor
Viruses	
Adenovirus (E3 region)	Adenovirus
Avian Leukosis Virus	Causes avian leucosis
Bovine Leukemia Virus	Causes bovine leukaemia
CMV	Cytomegalovirus
EBV(Wp promoter)	Epstein-Barr virus
HBV (pregenomic promoter)	Hepatitis B virus
HIV-1	Human immunodeficiency virus
HSV	Herpes simplex virus
JC Virus	Polyoma virus
HPV type 16	Human papillomavirus
SIV	Simian immunodeficiency virus
SV-40	Simian virus 40
Enzymes	
*ABC Transproters	ATP-binding membrane transporters
*N-acetylglucosaminyltransferase I (rat gene)	N-acetylglucosaminyltransferase
ADH	Liver alcohol deydrogenase
ARF-related protein-1	GTPase
Aromatase (promoter II)	Estrogen synthesis
α 1ACT	Anti-chymotrypsin
*BACE	β site APP cleaving enzyme

Cathepsin B	Lysosomal cysteine protease
*Cathepsin L	Lysosomal cysteine protease
*Ceramide glycoxyltransferase	Glycosphingolipid
*cis-retinoid/androgen dehydrogenase type 1 and 2 (CRAD 1 and CRAD2)	Short chain dehydrogenase
Collagenase 1	Matrix metalloproteinase
*Dihydrodiol dehydrogenase	Oxidoreductase, oxidation of trans-hydodiols
*DYPD	Dihydropyrimidine dehydrogenase
DNASIL2	DNAse-like endonuclease
*ENO2	Enolase 2γ
*GAD67	Glutamic acid decarboxylase
CD3-synthase	Sialyltransferase
Gelatinase B	Matrix metalloproteinase
GSTP1-1	Glutathione S-transferase
Glutamate-cysteine ligase	Glutathione S-transferase synthesis enzyme
GCLC	Glutamate-cysteine ligase catalytic subunit
*Glutamate-cysteine ligase modifier	Glutathione S-transferase synthesis enzyme
*Glucose I-6-phosphate dehydrogenase	Hexose monophosphate
*Soluble Guanylyl cyclase α (1)	Receptor for NO
*Heparanase	Cleaves heparin
HO-1	Hemeoxygenase
Hyaluronan synthase	Synthesizes hyluronic acid
11βHSD2	11β-hydroxysteroid dehydrogenase type 2
H+-K+-ATPase α2	role in potassium homeostasis
Iodothyronine deiodinase (type 2)	converts T4 to T3
Lipocalin-type prostaglandin D synthase (L-PGDS)	Prostaglandin D2 synthase in brain
Lysozyme	Hydrolyzes bacterial cell walls
*MKP-1	MAP kinase phosphatase
MMP-3, matrix metalloproteinase-3	Secreted collagenase involved in metastasis
MMP-9, matrix metalloproteinase-9	Secreted collagenase involved in metastasis
iNOS	Inducible nitric oxide synthase
*PDE7A1	Phosphodiesterase 7A1
PIM-1	Ser/Thr kinase
Plk3	Polo-like (Ser/Thr) kinase 3
*PP5	Protein phosphatase 5
PKCδ	Protein kinase D isoform
PLCδ1	Phospholipase C isoform
*PTGIS, prostaglandin synthase	Prostaglandin synthase
*PGES, prostaglandin E synthase	Prostaglandin synthase
RACK1	Receptor for activated C kinase
*REV3	DNA polymerase zeta
Serpin 2A	Serine protease
SIAT1	Sialyltransferase
SNARK	SNF1/AMPK-related kinase
TERT (mouse)	Telomerase catalytic subunit
Transglutaminase	Forms isopeptide bonds
Type II-secreted phospholipase A2	Proinflammatory phospholipase
*Xanthine dehydrogenase	Oxidative metabolism of purines
Miscellaneous	
α-1 acid glycoprotein	Serum protein
α-fetoprotein	Liver cancer marker
AMH	Anti-mullerian hormone
*β-amyloid	Alzheimer's precursor
Apolipoprotein C III	Apoprotein of HDL
*Apolipoprotein E	Protein assoc. with Alzheimers
*Biglycan	Connective tissue proteoglycan
BRCA2	Breast cancer susceptibility protein-2

*Caveolin-1	Lipid raft protein
*Clone 330	Possible secreted protein
*Clone 156	Unknown NF-κB inducer
*Clone 68	Unknown NF-κB inducer
*p21-CIP1	Cyclin-dependent kinase inhibitor
*Claudin-2	Gap junction protein
α2(I) collagen	Type I collagen
*Connexin32	Gap junction protein
Cyclin D1	Cell-cycle regulation
Cyclin D2	Cell-cycle regulation
*Cyclin D3	Cell-cycle regulation
Ephrin-A1	Cell-cycle regulation
Factor VIII	Hemostasis
Gadd45β	DNA repair/cell cycle
Gα i2	G protein
*GIF	Cys-rich metal binding protein
Galectin 3	β-galactosidase-binding lectin
GBP-1	GTPase guanylate binding protein
ε-Globin	Globin protein
*GS3686	Homology to microtubule aggragating protein
HMG 14	High mobility group 14
K3 Keratin	Intermediate filament protein
K6 Keratin	Intermediate filament protein
K15 Keratin	Intermediate filament protein
Laminin B2 chain	Basement membrane protein
Mts1	Multiple tumor suppressor
MNE1	Monocyte/neutrophil elastase inhibitor
Mucin (MUC-2)	Airway defense glycoprotein
Mx1 (bovine)	Viral resistance
Neutrophil gelatinase-associated lipocalin	Anti-microbial protein in lung
NLF1	IL-1β induced
*p11	Annexin II ligand
PAI-1	Plasminogen activator inhibitor
*Pax8	Paired box gene
*PCBC	6-pyruvoyl-tetrahydropterin synthase
Perforin	Pore-forming effector molecule
*PGK1	Phosphoblycerate kinase 1
POMC	Proopiomelanocortin
Pregnancy-specific glycoprotein rnCGM3	Placental expression
Prodynorphin	Neuropeptide
Prostate-specific antigen	Serum protein in prostate cancer
*RICK	Adaptor
S100A6 (calcyclin)	Calcium binding protein
*Spergen-1	Sperm-speicific mitochondrial protein
SWS1	Rainbow trout opsin
Syndecan-4	Heparin sulfate proteoglycan
*Tissue factor pathway inhibitor-2 (TFPI-2)	Serine protease inhibitor
*Transferrin (mosquito)	Probable iron transport protein
TRIF	TIR-containing adaptor protein inducing interferon β
*UBE2M	Ubiquitin conjugating enzyme E2M
*UCP-2	Uncoupling protein-2
Uroplakin Iβ	Surface structural protein on urothelial cells
25-hydroxyvitamin D3 1-α hydroxylase	Enzyme for liver vitamin D3 production
Vimentin	Intermediate filament protein
α1-antitrypsin	Protease inhibitor

9. Annex B:

- The EMBO Journal (2005) 24, 85-96: Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-κB-dependent gene expression
- The Journal of Biological Chemistry (2005) Vol. 280, No. 49, p. 40450-40464: Acetylation of Poly(ADP-ribose) Polymerase-1 by p300/CREB-binding Protein Regulates Coactivation of NF-κB-dependent Transcription

The EMBO Journal (2005) 24, 85–96 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
www.embojournal.org

THE EMBO JOURNAL

Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-κB-dependent gene expression

Marcela Covic[1,4], Paul O Hassa[1,4], Simona Saccani[2], Christine Buerki[1], Nadja I Meier[1], Cornelia Lombardi[1], Ralph Imhof[1], Mark T Bedford[3], Gioacchino Natoli[2] and Michael O Hottiger[1,*]

[1]Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Zurich, Switzerland, [2]Institute for Research in Biomedicine, Bellinzona, Switzerland and [3]Department of Carcinogenesis, University of Texas, Smithville, TX, USA

Nuclear factor kappaB (NF-κB) plays an important role in the transcriptional regulation of genes involved in inflammation and cell survival. Here, we show that coactivator-associated arginine methyltransferase CARM1/PRMT4 is a novel transcriptional coactivator of NF-κB and functions as a promoter-specific regulator of NF-κB recruitment to chromatin. Carm1 knockout cells showed impaired expression of a subset of NF-κB-dependent genes upon TNFα or LPS stimulation. CARM1 forms a complex with p300 and NF-κB *in vivo* and interacts directly with the NF-κB subunit p65 *in vitro*. CARM1 seems to act in a gene-specific manner mainly by enhancing NF-κB recruitment to cognate sites. Moreover, CARM1 synergistically coactivates NF-κB-mediated transactivation, in concert with the transcriptional coactivators p300/CREB-binding protein and the p160 family of steroid receptor coactivators. For at least a subset of CARM1-dependent NF-κB target genes, the enzymatic activities of both CARM1 and p300 are necessary for the observed synergy between CARM1 and p300. Our results suggest that the cooperative action between protein arginine methyltransferases and protein lysine acetyltransferases regulates NF-κB-dependent gene activation *in vivo*.

The EMBO Journal (2005) **24,** 85–96. doi:10.1038/sj.emboj.7600500; Published online 16 December 2004
Subject Categories: chromatin & transcription
Keywords: CARM1; ChIP; p300; p65/RelA; methylation

Introduction

Nuclear factor kappaB (NF-κB) is a widely expressed, inducible transcription factor, which plays a key role in the transcriptional regulation of a variety of genes involved in mammalian immune and inflammatory responses (Ghosh *et al*, 1998). NF-κB has additionally been implicated as an important regulator of cellular events such as apoptosis, cell proliferation and differentiation (Baldwin, 1996). NF-κB is composed of members of the Rel family, which in eukaryotes includes p50/p105 (NF-κB1), p52/p100 (NF-κB2), Rel (c-Rel), p65 (RelA) and RelB (Ghosh *et al*, 1998). These proteins share a conserved 300 amino-acid region within their amino-termini, known as the Rel-homology domain (RHD), which mediates dimerization, nuclear translocation, DNA-binding and interaction with heterologous transcription factors and NF-κB inhibitors (Ghosh *et al*, 1998). The specificity of NF-κB-dependent transcription is thought to be at least partially due to differential homo- and heterodimerization of its family members, leading to a range of DNA-binding and activation potential (Karin, 1998). The most abundant and best-studied form of NF-κB in cells is a 'classical' heterodimer consisting of the two subunits p50 (NF-κB1) and p65 (RelA). Although all Rel family members bind to DNA, only p65 (RelA), Rel (c-Rel) and RelB contain a transactivation domain. In most differentiated unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor complex by its physical association with one of the several inhibitors of NF-κB (IκB) (Baeuerle and Baltimore, 1988; Whiteside and Israel, 1997). Treatment of cells with extracellular stimuli such as cytokines, bacterial lipopolysaccharides (LPS) or potent oxidants leads to the rapid phosphorylation of the IκBs, which results in their ubiquitination and subsequent degradation by the 26S-proteasome pathway (Karin, 1998; Karin and Ben-Neriah, 2000). Consequently, NF-κB accumulates in the nucleus, binds to specific κB consensus sequences in the chromatin and activates specific subsets of genes.

The assembly of a higher order NF-κB transcription complex is an important stage in NF-κB-dependent transcription, involving multiple coactivator/cofactor–NF-κB–DNA interactions (Merika *et al*, 1998; Agalioti *et al*, 2000; Merika and Thanos, 2001). The two key coactivators of NF-κB, histone-acetyltransferases p300 and its homolog, the CREB-binding protein (CBP), directly associate with the NF-κB subunits p50 and p65 (Gerritsen *et al*, 1997; Perkins *et al*, 1997; Hassa *et al*, 2003). These coactivators are thought to promote the rapid formation of the pre-initiation and re-initiation complexes by bridging the sequence-specific activators (like NF-κB) to the basal transcriptional machinery, thereby facilitating multiple rounds of transcription (Goodman and Smolik, 2000). Additionally, the histone acetyltransferases p300 and CBP can modify the amino-terminal tails of nucleosomal histones, thereby altering the local chromatin structure (Schiltz *et al*, 1999; Kundu *et al*, 2000). It was proposed that the coactivator p300/CBP functions also as a signal integrator by coordinating diverse signal transduction events at the transcriptional level (Goodman and Smolik, 2000). Phosphorylation of p65 by protein kinase A (PKA) has been shown to stimulate NF-κB-dependent gene expression by enhancing p65 association with p300/CBP (Zhong *et al*, 1998). Previous reports have

*Corresponding author. Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. Tel.: +41 1 635 5474; Fax: +41 1 635 6840; E-mail: hottiger@vetbio.unizh.ch
[4]These two authors contributed equally to this work

Received: 9 July 2004; accepted: 9 November 2004; published online: 16 December 2004

shown that NF-κB-dependent transcriptional complexes also require the p300/CBP-associated factor (P/CAF), the p160 family of steroid receptor coactivators and PC1/PARP-1 (Sheppard *et al*, 1999; Hassa *et al*, 2001, 2003). Thus, additional components might be required to stabilize the association of distinct NF-κB coactivator complexes.

Coactivator-associated arginine methyltransferase (CARM1/PRMT4) was identified as SRC-2/TIF2/GRIP1-binding protein and belongs to a family of arginine-specific protein methyltransferases, which includes at least seven members (PRMT1–7) (McBride and Silver, 2001; Miranda *et al*, 2004). CARM1 has been shown to synergistically stimulate transcription by nuclear receptors in combination with the p160 family of coactivators and forms a ternary complex with p300/CBP and SRC-2/TIF2/GRIP1 (Koh *et al*, 2001; Lee *et al*, 2002). After recruitment to the promoters of estrogen-responsive genes, CARM1 methylates specific arginine residues (Arg17 and Arg26) in the N-terminal tail of histone H3 as part of the transcriptional activation process (Bauer *et al*, 2002; Daujat *et al*, 2002). Recent studies broadened the targets of the transcriptional coactivator function of CARM1: CARM1 coactivates p53-dependent transcription and cooperates with β-catenin to enhance transcriptional activation by the lymphoid enhancer factor/T-cell factor (LEF1/TCF4) (Koh *et al*, 2002; An *et al*, 2004). Mice with a targeted disruption of Carm1 die during late embryonic development or immediately after birth, supporting the idea that CARM1 is a crucial coactivator for gene expression during late embryonic development (Yadav *et al*, 2003). Aberrant T-cell development in Carm1-deficient embryos was due to a partial developmental arrest in the earliest thymocyte progenitor subset, indicating that CARM1 plays a significant role in promoting the differentiation of early thymocyte progenitors (Kim *et al*, 2004).

Since the transcriptional coactivator CARM1 is capable of forming a complex with the known coactivators of NF-κB, p300/CBP and SRC-2, we tested whether CARM1 participates in NF-κB-dependent gene activation. Our results show that CARM1 directly binds to the NF-κB subunit p65 and synergistically coactivates NF-κB-mediated transactivation, in concert with the transcriptional coactivators p300/CBP and the p160 family of steroid receptor coactivators. CARM1 is responsible for H3(R17) methylation of NF-κB target genes *in vivo* and is required for NF-κB-regulated activation of a subset of genes. CARM1 functions by enhancing NF-κB recruitment to κB sites contained in H3(R17)-methylated promoters, although additional roles downstream of p65 recruitment mediated by its bridging factor/coactivator activity cannot be ruled out. These results suggest that the cooperative action between coactivators with histone arginine methyltransferase activities and at least two distinct classes of transcription coactivator molecules with histone acetyltransferase activities regulates NF-κB-dependent gene activation *in vivo*.

Results

NF-κB-dependent gene expression is impaired in Carm1(−/−) cells

To test whether CARM1 influences NF-κB-dependent gene expression, Carm1(+/+) or Carm1(−/−) MEFs were treated with LPS as indicated and the expression of NF-κB-dependent genes was assessed by RT–PCR (Figure 1A). The experiments

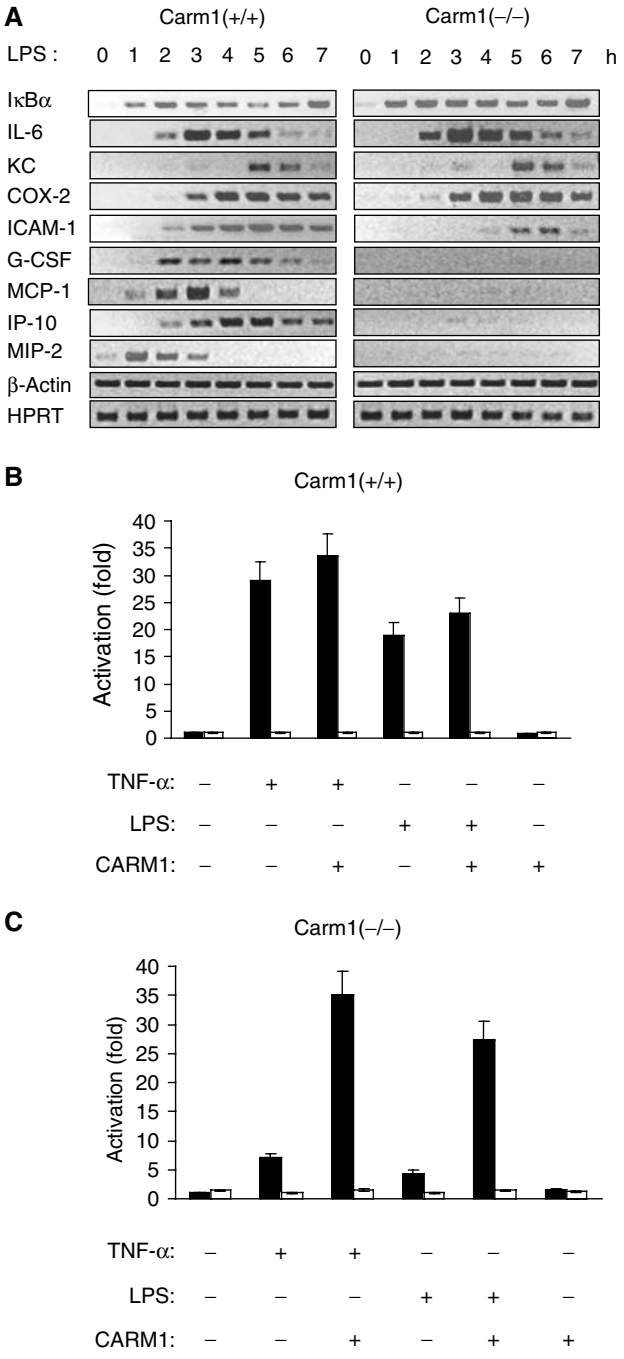


Figure 1 Impaired NF-κB-dependent gene expression in Carm1(−/−) cells in response to proinflammatory stimuli. (**A**) Impaired expression of MIP-2, MCP-1, G-CSF, ICAM1 and IP-10 in Carm1(−/−) MEF cells in response to LPS. Carm1(+/+) and Carm1(−/−) MEF cells were treated with LPS (10 μg/ml) and RNA isolated at the indicated time points, followed by RT–PCR determination of MIP-2, MCP-1, G-CSF, ICAM1, IP-10, IL-6, KC, COX-2, HPRT and β-actin mRNA. (**B, C**) Impaired NF-κB-dependent gene expression in Carm1(−/−) cells in response to TNFα and LPS. Carm1(+/+) MEF cells (B) and Carm1(−/−) MEF cells (C) were cotransfected with HIV-luc or HIVmut-κB-luc (2 μg) and pphRSVnt-β-gal (200 ng) together with CMV-CARM1 (500 ng) or CMV empty vector as indicated. Cells were subsequently stimulated with TNFα (10 ng/ml) or LPS (10 μg/ml) 24 h after transfection for 8 h. The indicated activation was determined by the ratio of the relative luciferase activity measured for the HIV-luc (black bars) or HIVmut-κB-luc (white bars) reporter gene after stimulation. The ratio obtained for untreated cells was arbitrarily set to 1. Error bars indicate s.e. of three independent experiments.

revealed that LPS-induced levels of G-CSF, MIP-2, MCP-1, ICAM1 and IP-10 were impaired in Carm1(−/−) cells (Figure 1A). However, the expression of IκBα, IL-6, KC and COX-2 was not reduced (Figure 1A), indicating that only a subset of NF-κB-dependent genes requires CARM1 for induction. The expression of IκBα and IL-6 was even slightly upregulated in Carm1(−/−) cells, a result confirmed by quantitative real-time PCR (Figure 1A and data not shown). Similar results were obtained when cells were stimulated with TNFα (see below). To further investigate the relevance of CARM1 in NF-κB-dependent gene expression, Carm1(+/+) or Carm1(−/−) cells were transfected with the indicated NF-κB-dependent luciferase reporter constructs together with an expression vector for CARM1, and subsequently treated with the indicated stimuli (TNFα or LPS). Cell lysates were tested for luciferase activity as a measure of NF-κB activity (Figure 1B and C). Although TNFα and LPS were able to induce NF-κB-dependent transcriptional activation in Carm1(+/+) cells (Figure 1B), the NF-κB-dependent transcriptional activation was severely reduced in Carm1(−/−) cells upon stimulation with TNFα or LPS (Figure 1C). Re-expression of CARM1 in Carm1(−/−) cells synergistically enhanced NF-κB-dependent transcriptional activation five-fold in response to TNFα or LPS (Figure 1C). No synergistic activation was observed in Carm1(+/+) cells, indicating that endogenous CARM1 levels are sufficient to provide maximal coactivation (Figure 1B). The same transfection experiments with a reporter gene under the control of mutated κB sites revealed that the observed induction was NF-κB-specific. Together, these results indicate that CARM1 is required for NF-κB-dependent transactivation of extra-chromosomal templates upon stimulation with proinflammatory stimuli.

Normal NF-κB induction in Carm1(−/−) cells

To analyze whether the activation of the NF-κB-signaling pathway is affected in Carm1(−/−) cells upon stimulation with TNFα or LPS, we assessed the expression levels of important components of the NF-κB-signaling pathway by immunoblot and RT–PCR analysis (Figure 2A and B). The immunoblot analysis of nuclear and cytoplasmic extracts of Carm1(+/+) and Carm1(−/−) cells revealed that p65, c-Rel, RelB, p300 and PARP-1 are equally expressed in Carm1(+/+) and Carm1(−/−) cells (Figure 2A, left and right panels). Additionally, these experiments showed that there is equivalent nuclear translocation of NF-κB in Carm1(+/+) and Carm1(−/−) cells. Moreover, the expression levels of upstream signaling components, TLR-2, TLR-4 and IRAK4 mRNA, was not impaired in Carm1(−/−) cells (Figure 2B, right panel), compared to Carm1(+/+) cells (Figure 2B, left panel). We then investigated whether the degradation of IκBα might be affected due to the absence of CARM1. Carm1(+/+) and Carm1(−/−) cells were treated with TNFα or LPS for the indicated time points and the protein levels of IκBα subsequently tested by immunoblot analysis (Figure 2C). No differences in degradation and re-synthesis of IκBα could be observed between Carm1(+/+) and Carm1(−/−) cells upon stimulation with TNFα (Figure 2C) or LPS (data not shown). To further investigate whether the activation of the NF-κB-signaling pathway is affected in Carm1(−/−) cells upon stimulation, we analyzed the kinetics of nuclear translocation of p65 in Carm1(+/+) and Carm1(−/−) cells by immunofluorescence analysis. Kinetics of p65 nuclear entry in Carm1(−/−) cells was comparable to that observed in Carm1(+/+) cells (Figure 2D). Finally, we tested whether the DNA-binding activity of NF-κB on naked templates is affected in

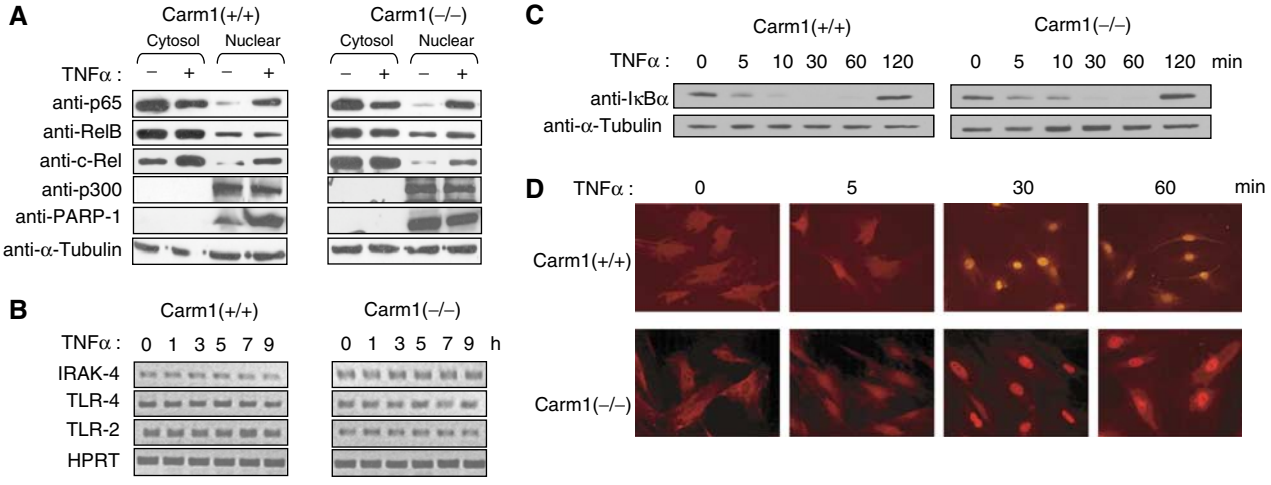


Figure 2 Expression levels of components of the NF-κB signaling pathway and nuclear–cytoplasmic shuttling of NF-κB is not affected in Carm1(−/−) MEFs. (**A**) Protein expression levels of p65, c-Rel, RelB, p300 and PARP-1 are not affected in Carm1(−/−) MEF cells. Carm1(+/+) and Carm1(−/−) MEF cells were treated with TNFα (10 ng/ml) for 20 min and cytoplasmic and nuclear extracts resolved by SDS–PAGE followed by subsequent immunoblot analysis for p65, c-Rel, RelB, p300 and PARP-1 (A, left and right panels). α-Tubulin was used as an internal standard. (**B**) mRNA expression levels of TLR4, TLR2 and IRAK4 are not impaired in Carm1(−/−) MEF cells. Carm1(+/+) and Carm1(−/−) MEF cells were treated with TNFα (10 ng/ml) (B, left and right panels) and RNA isolated at the indicated time points followed by RT–PCR analysis for TLR4, TLR2, IRAK4 and HPRT (B, left and right panels). (**C**) Degradation and re-synthesis of IκBα is not affected in Carm1(−/−) MEF cells. Carm1(+/+) and Carm1(−/−) MEF cells were treated with TNFα (10 ng/ml) (C, left and right panels) and whole-cell extracts isolated at the indicated time points and resolved by SDS–PAGE, followed by subsequent immunoblot analysis for IκBα. α-Tubulin was used as an internal standard. (**D**) Nuclear translocation of NF-κB is not delayed in Carm1(−/−) MEFs. Carm1(+/+) and Carm1(−/−) MEF cells were treated with TNFα (10 ng/ml) (D, upper and lower panels) and fixed with paraformaldehyde at the indicated time points followed by immunostaining for p65 and analysis by immunofluorescent microscopy.

Carm1(−/−) cells. EMSA studies with DNA oligos corresponding to the κB sites in IL-6, IP-10 and MIP-2 promoter revealed no differences between Carm1(+/+) and Carm1(−/−) cells in DNA-binding activity of NF-κB (p50/p65) to nonchromatinized templates (data not shown).

CARM1 forms a complex with NF-κB in vivo and binds directly to p65 in vitro

To investigate whether CARM1 physically interacts with NF-κB *in vivo*, we immunoprecipitated CARM1 complexes from nuclear extracts upon stimulation of 293T cells with TNFα and tested the presence of p65 and p300 by immunoblot analysis using anti-p65, anti-CARM1 or anti-p300 antibodies. p65 and p300 formed a complex with CARM1 in the nucleus (Figure 3A). DNA did not mediate the association of p300 and p65 with CARM1 in the nucleus since the presence of ethidium bromide or DNAse1 did not affect p300/p65/CARM1 binding (data not shown). As these results strongly suggested that CARM1 would directly interact with at least one subunit of NF-κB, recombinant purified GST-p65 full length was bound to glutathione beads, followed by incubation with recombinant purified full-length His-CARM1 (Figure 3B). After extensive washes, bound proteins were resolved by SDS–PAGE, followed by immunoblot analysis for CARM1. CARM1 was able to bind directly to the NF-κB subunit p65 but not to the GST control (Figure 3B). To map the interaction domains within p65 and CARM1, GST and GST-fusion proteins expressing either the Rel-homology domain of p65 (RHD; aa 1–305), or the transactivation domain of p65 (aa 441–551), were used in GST pull-down experiments with *in vitro* translated full-length CARM1 or deletion mutants corresponding to the N-terminal domain (aa 1–148), catalytic domain (aa 140–480) or transactivation domain (aa 462–608), respectively. These experiments revealed that a region between aa 148 and 462 containing the catalytic domain but not the N- or C-terminal domain of CARM1 was able to selectively interact with the RHD of p65 (Figure 3C), confirming that the interactions described above were direct and not mediated by other proteins.

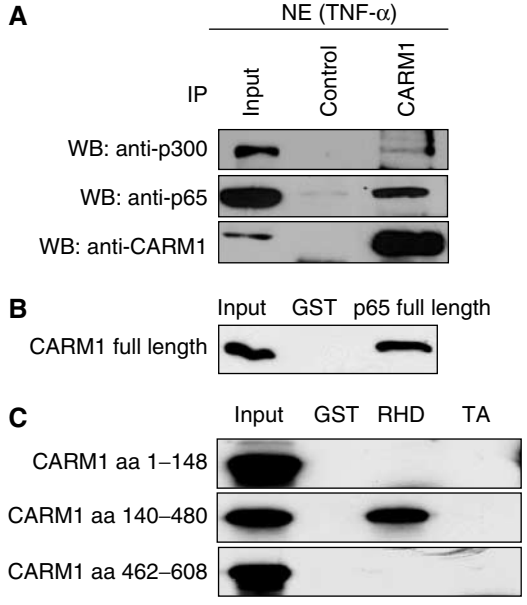


Figure 3 CARM1 forms a complex with NF-κB *in vivo* and directly binds to the NF-κB subunit p65 *in vitro*. (**A**) CARM1 and p65 form a complex *in vivo*. p65, CARM1 and p300 were coimmunoprecipitated (IP) in the presence of 120 mM NaCl from nuclear extract of TNFα-treated 293T cells (30 min, 10 ng/ml) using control IgGs and an anti-CARM1 antibody. Bound proteins were resolved by SDS–PAGE and subsequently detected by immunoblot (IB) analysis for p65, CARM1 and p300. Input lanes represent 10% of the input. (**B**) CARM1 directly binds to p65. Pull-down assays with purified GST and p65 full-length fused to GST (1 μg) and purified recombinant CARM1 full-length (0.1 μg) in the presence of 120 mM NaCl. Bound proteins were resolved by SDS–PAGE and detected by immunoblot analysis for CARM1. Input lanes represent 1% of the input. (**C**) The central domain of CARM1 binds to the RHD domain of p65. Pull-down assays with GST, the Rel-homology domain of p65 (RHD; aa 1–305) or the transactivation domain of p65 (TA; aa 441–551) fused to GST (1 μg) and different *in vitro*-translated CARM1 deletion mutants (N-terminal domain (aa 1–148), catalytic domain (aa 140–480) or transactivation domain (aa 462–608), respectively) in the presence of 120 mM NaCl. Bound proteins were resolved by SDS–PAGE visualized by autoradiography. Input lanes represent 1% of the input.

CARM1 coactivates NF-κB synergistically with p300/CBP

CARM1 and p300/CBP were recently shown to form a ternary complex and to function synergistically to enhance the activity of nuclear receptors (Lee *et al*, 2002). Thus, CARM1 in combination with p300 might also synergistically coactivate NF-κB-mediated transactivation. In order to directly test this possibility, we transfected Carm1(+/+) and Carm1(−/−) cells with expression vectors for CARM1 and p300 along with a NF-κB-dependent luciferase reporter; cells were subsequently treated with TNFα or LPS (Figure 4A and B). p300 and CARM1 synergistically stimulated NF-κB-mediated transcription in response to TNFα or LPS (Figure 4A and B). Expression of p300 synergistically enhanced reporter gene expression (up to three-fold) in Carm1(+/+) cells in response to TNFα or LPS (Figure 4A and B, left panel). However, in Carm1(−/−) cells, the synergy between p300 and TNFα or LPS was severely impaired (Figure 4A and B, right panel). Coexpression of CARM1 together with p300 caused a highly synergistic enhancement in Carm1(−/−) cells (Figure 4A and B, right panel), indicating that the presence of CARM1 itself is required for the synergistic transcriptional activation of NF-κB by p300 (Figure 4A and B).

To confirm these results, experiments were repeated overexpressing p65 without exogenous stimulation. The amount of p65 expression vector strongly influenced the degree of cooperation among these coactivators; as previously shown for nuclear receptors (Lee *et al*, 2002), high synergistic enhancement of transcriptional coactivation was obtained only under conditions where limiting levels of p65 were expressed (data not shown). Therefore, we decided to proceed with low amounts of p65 expression vectors for all the following experiments. Carm1(+/+) and Carm1(−/−) cells were transfected with expression vectors for CARM1, p65 and p300 along with a NF-κB-dependent luciferase reporter containing wild-type or mutated κB sites. In the absence of cotransfected coactivator, the low levels of p65 used produced only a slight increase (4.5-fold) in luciferase activity in Carm1(+/+) cells (Figure 4C, left panel). No significant increase (1.5-fold) in luciferase activity could be observed in Carm1(−/−) cells (Figure 4C, right panel). Coexpression of CARM1 with p65 augmented NF-κB-dependent transcrip-

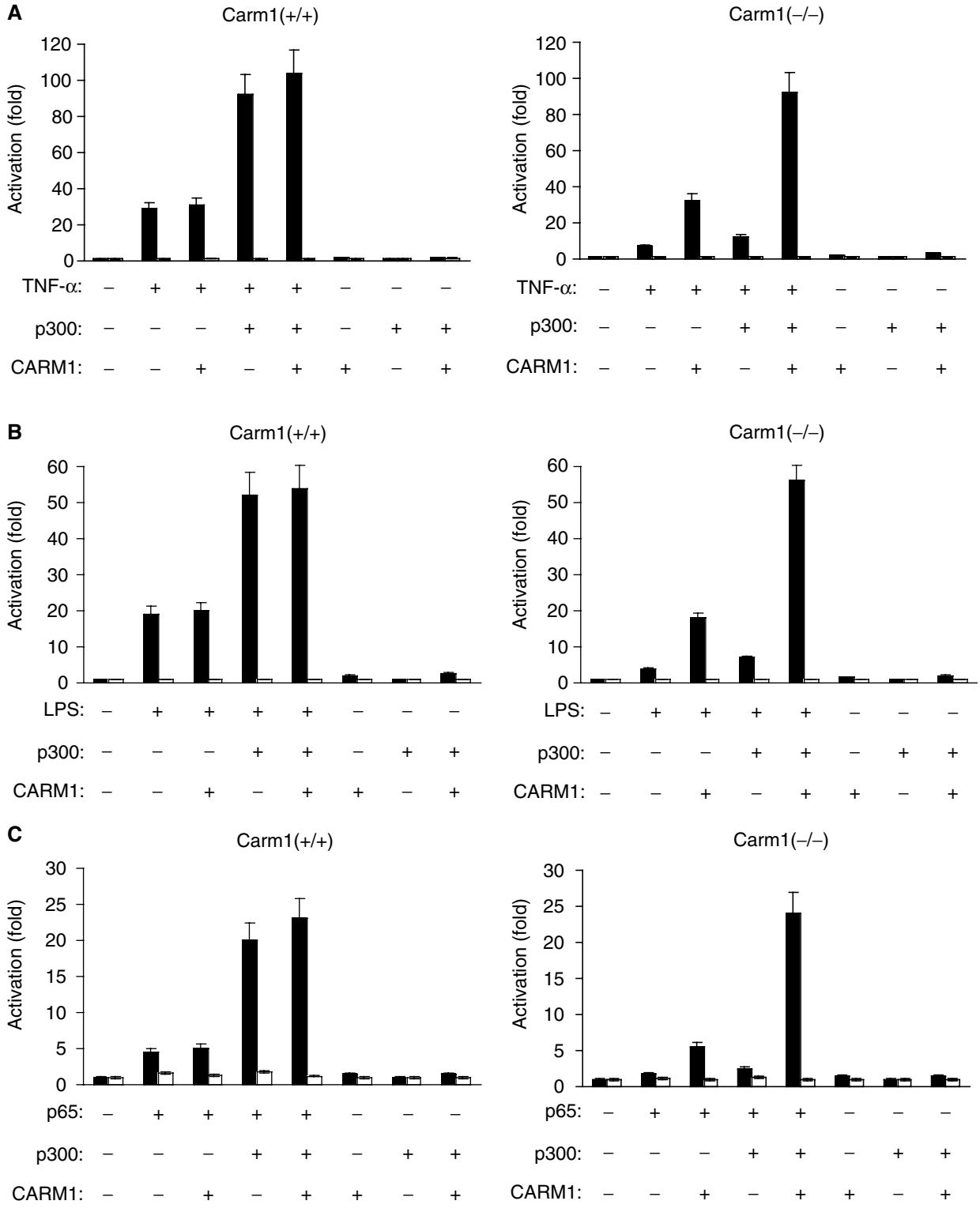


Figure 4 CARM1 synergistically coactivates the NF-κB-mediated transactivation, in concert with the transcriptional coactivator p300/CBP. (**A, B**) CARM1 synergistically activates together with p300 NF-κB-dependent gene expression in response to proinflammatory stimuli. Carm1(+/+) MEF cells (left panel) and Carm1(−/−) MEF cells (right panel) were cotransfected with HIV-luc or HIVmut-κB-luc (2 μg) and pphRSVnt-β-gal (200 ng), together with CMV-CARM1 (200 ng) and RSV-p300 (500 ng) or CMV and RSV empty vectors as indicated. Cells were subsequently stimulated with TNFα (10 ng/ml) (A) or LPS (10 μg/ml) (B) 24 h after transfection for 8 h. Cells were harvested 32 h after transfection and the indicated activation of NF-κB-dependent gene expression determined as described in Figure 1. (**C**) CARM1 synergistically activates together with p300 and p65-dependent gene expression. Carm1(+/+) MEF cells (left panel) and Carm1(−/−) MEF cells (right panel) were cotransfected with HIV-luc or HIVmut-κB-luc (2 μg) and pphRSVnt-β-gal (200 ng) together with CMV-p65 (80 ng) CMV-CARM1 (200 ng) and RSV-p300 (500 ng) or CMV and RSV empty vectors as indicated. The indicated activation of NF-κB-dependent gene expression was determined as described in Figure 1.

tional activation in Carm1(−/−) cells to a similar extent as observed in Carm1(+/+) cells with p65 alone (Figure 4C, left panel). Coexpression of p300 with p65 synergistically enhanced reporter gene expression (four-fold) in Carm1(+/+) cells (Figure 4C, left and right panels), whereas no synergistic enhancement could be observed in Carm1(−/−) cells (Figure 4C, right panel). However, addition of CARM1 with p300 caused a highly synergistic enhancement in Carm1(−/−) cells (Figure 4C, right panel). No synergistic activation was observed in Carm1(+/+) cells, indicating that endogenous CARM1 is sufficient to provide maximal coactivation (Figure 4C, left panel). When p300 was substituted for CBP, a similar level of synergy was observed (data not shown).

CARM1 and SRC-2/TIF2/GRIP1 synergistically coactivate NF-κB-mediated transactivation

Next, we tested whether the combined coexpression of p65 together with CARM1, p300 and p160 family members might result in even stronger synergistic coactivation of NF-κB. Coexpression of SRC-2/TIF2/GRIP1 with p65 and p300 resulted in a additional (three-fold) synergistic increase in NF-κB activity in Carm1(+/+) cells (Figure 5A), compared to coexpression of p65 and p300. This could not be observed in Carm1(−/−) cells (Figure 5B). This synergy between p300 and p160 family members observed in Carm1(+/+) cells is in agreement with recent reports, showing that NF-κB-dependent transcriptional activity requires p300/CBP but also the p160 family of steroid receptor coactivators (Sheppard *et al*, 1999). Remarkably, strong synergistic coactivation of NF-κB by p300 and SRC-2/TIF2/GRIP1 could be observed in Carm1(−/−) cells only when CARM1 was present (Figure 5B), while additional expression of CARM1 did not enhance NF-κB-dependent transcriptional activation in Carm1(+/+) cells (Figure 5A). Combined coexpression of p65 together with CARM1, p300 and other p160 family members in Carm1(−/−) cells led to similar results, although the additional synergistic coactivation of NF-κB by SRC-1/NCoA-1 or SRC-3/p/CIP/RAC-3 was not as high as observed for SRC-2/TIF2/GRIP1 (data not shown). To further confirm the relevance of CARM1 for synergistic coactivation of NF-κB by p300 and p160 family members, we repeated these experiments in the monkey COS-1 cells (Figure 5C). The results of these experiments revealed that combined expression of p65 together with all three coactivators in COS-1 cells led to a similar level of coactivator synergy as observed in Carm1(−/−) cells (Figure 5C). Coexpression of CARM1 resulted in a strong synergistic coactivation, despite endogenous CARM1 expression in these cells. Thus, the endogenous levels of CARM1 seem to be limiting in these cells. Together, these results strongly suggest that CARM1 is required for synergistic coactivation of NF-κB-dependent gene activation by p300/CBP and p160 family members.

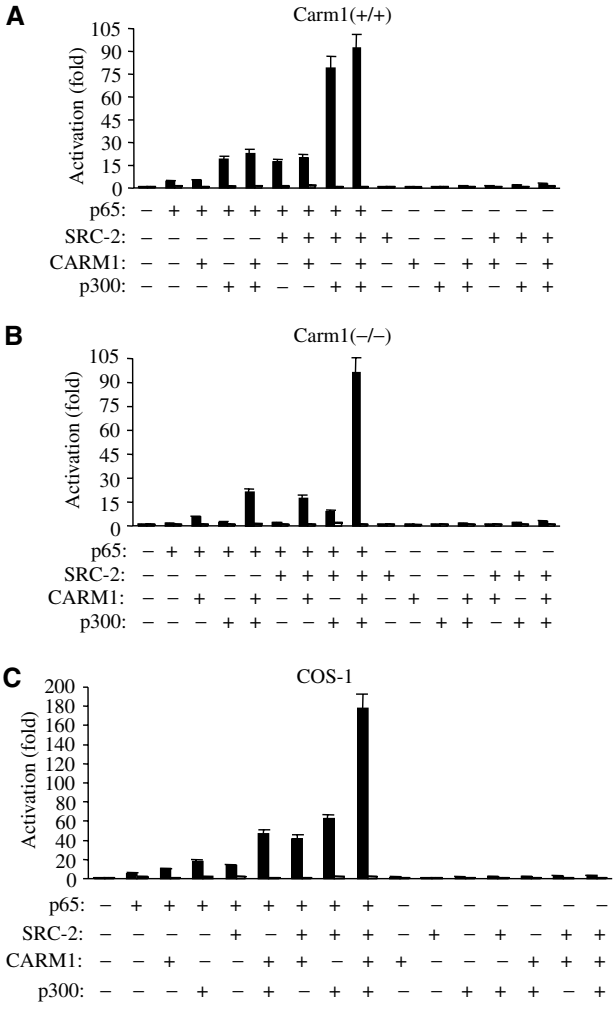


Figure 5 CARM1 synergistically coactivates NF-κB-dependent transcription in concert with the transcriptional coactivators p300/CBP and the p160 family of steroid receptor coactivators. (**A**, **B**) CARM1 synergistically activates together with p300 and SRC-2 p65-dependent gene expression. Carm1(+/+) MEF cells (A) and Carm1(−/−) MEF cells (B) were cotransfected with HIV-luc or HIVmut-κB-luc (2 μg) and pphRSVnt-β-gal (200 ng), together with CMV-p65 (80 ng), CMV-CARM1 (200 ng), CMV-SRC-2 (300 ng) and RSV-p300 (500 ng) or CMV and RSV empty vectors as indicated. Cells were harvested 32 h after transfection and the indicated activation of NF-κB-dependent gene expression determined as described in Figure 1. (**C**) CARM1 synergistically activates together with p300 and p65 NF-κB-dependent gene expression in COS-1 cells. COS-1 cells were cotransfected with HIV-luc or HIVmut-κB-luc (2 μg) and pphRSVnt-β-gal (200 ng) together with CMV-p65 (50 ng) CMV-CARM1 (200 ng), CMV-SRC-2 (300 ng) and RSV-p300 (500 ng) or CMV and RSV empty vectors as indicated. The indicated activation of NF-κB-dependent gene expression was determined as described in Figure 1.

CARM1 is required for H3(R17) methylation and recruitment of p65 to the MIP-2 and IP-10 promoters

We next tested both CARM1 and p65 recruitment as well as H3(R17) methylation at NF-κB-dependent genes using quantitative chromatin immunoprecipitation (ChIP) assays. First, we confirmed by real-time PCR the CARM1 dependency of NF-κB-dependent gene induction in TNFα-stimulated cells. While MIP-2, IP-10 and MCP-1 mRNA induction in response to TNFα was nearly completely abrogated in Carm1(−/−) cells, IL-6 mRNA expression was not affected or even increased in Carm1(−/−) cells, thus strengthening the concept that CARM1 requirement is strictly gene-specific (Figure 6A). An anti-CARM1 ChIP assay revealed that CARM1 is not only recruited to the CARM1 dependent MIP-2 and IP-10 promoters but also to the CARM1 independent IL-6 promoter in Carm1(+/+) cells, upon stimulation (Figure 6B). We next investigated H3(R17) methylation at all three genes using a ChIP assay with an antibody recognizing H3(R17) only when methylated. A strong increase in H3(R17) methylation was

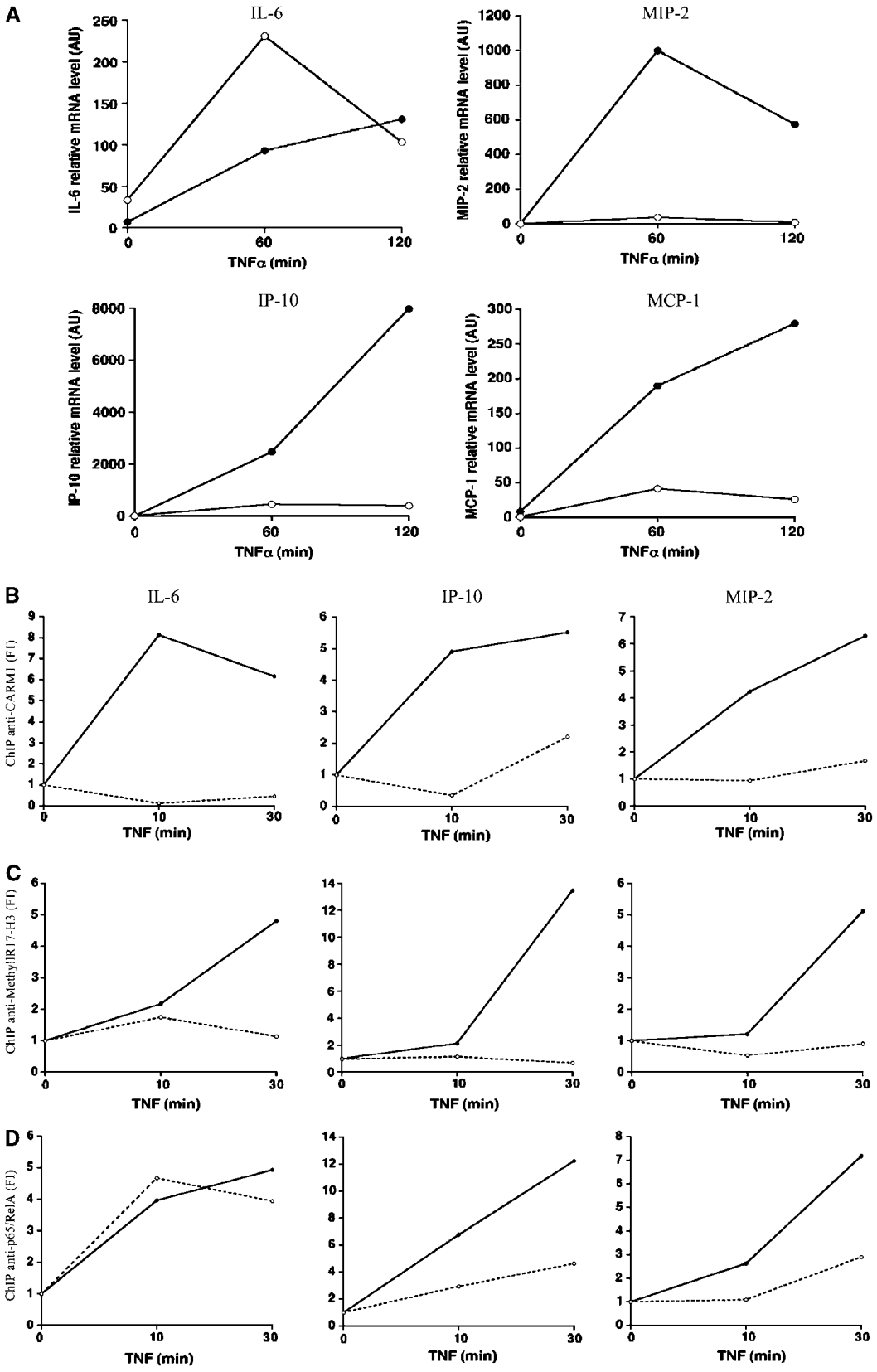


Figure 6 CARM1 functions in a gene-specific manner. (**A**) Quantification of TNFα-stimulated MIP-2, IP-10, MCP-1 and IL-6 transcription Carm1(+/+) MEF cells (filled circles) and Carm1(−/−) MEF cells (empty circles) by real-time PCR. (**B**) Quantitative ChIP assays with anti-CARM1 antibodies were performed for the indicated genes on TNFα-stimulated Carm1(+/+) (filled circles) and Carm1(−/−) MEF cells (empty circles). (**C**) Anti-methyl-H3(R17) ChIP assay for the indicated genes on TNFα-stimulated Carm1(+/+) (filled circles) and Carm1(−/−) MEF cells (empty circles). (**D**) ChIP assays with anti-p65 antibodies were performed for the indicated genes on TNFα-stimulated Carm1(+/+) (filled circles) and Carm1(−/−) MEF cells (empty circles).

found to occur on MIP-2, IP-10 and IL-6 promoters in TNFα stimulated Carm1(+/+) cells (Figure 6C). No H3(R17) methylation could be detected on either gene in Carm1(−/−) cells, indicating that methylation of H3(R17) is entirely CARM1-dependent (Figure 6C). Surprisingly, an anti-p65 ChIP assay revealed a major defect in p65 recruitment to the MIP-2 and IP-10 promoters in Carm1(−/−) cells (Figure 6D), whereas no significant differences could be observed at IL-6 between Carm1(+/+) and Carm1(−/−) cells (Figure 6D), thus indicating that the mechanism underlying CARM1 requirement for transcriptional induction differs depending on the gene. While these results implicate that CARM1 functions in a gene-specific manner by enhancing NF-κB recruitment to regulatory sites, possibly through an increase in κB site accessibility, they do not rule out the possibility that further effects downstream of NF-κB recruitment also occur. Moreover, the results obtained with IL-6 indicate that H3(R17) methylation may not be required for the activity of all promoters at which it occurs.

The enzymatic activities of CARM1 and p300/CBP are required for full cooperativity and NF-κB-dependent transcription

Since our ChIP study revealed induced methylation of H3(R17) upon stimulation, the observed cooperativity between CARM1 and p300/CBP regarding NF-κB-dependent gene expression might require their enzymatic activities. We first tested whether in transient luciferase reporter assays the IL-6-, MIP-2 and IP-10 promoters are regulated in Carm1(+/+) and Carm1(−/−) cells, similarly as their endogenous counterparts (Figure 7A). These experiments revealed that all promoters requiring CARM1 for activity were similarly dependent on CARM1 when taken out of their natural context and tested in reporter assays. Re-expression of CARM1 in Carm1(−/−) cells fully rescued stimulus-induced activation of the MIP-2 and IP-10 promoter in Carm1(−/−) cells (Figure 7A). We next transfected Carm1(−/−) cells with expression vectors for p65, wild-type or enzymatic mutants of CARM1 and/or p300 along with a luciferase reporter under the control of the endogenous MIP-2 or IP-10 promoter (Figure 7B and C). Coexpression of wild-type CARM1 and p300 together with limiting levels of p65 in Carm1(−/−) cells caused a highly synergistic enhancement of transcription regulated from both MIP-2 and IP-10 promoters (Figure 7B and C). However, the cooperativity/synergy between p300 and CARM1 was severely impaired when an enzymatic mutant form of either CARM1 or p300 was coexpressed. Coexpression of an enzymatic mutant form of both CARM1 and p300 caused an even more severe reduction in p65-dependent activation of the IP-10 and MIP-2 promoter, indicating that the enzymatic activities of both CARM1 and p300/CBP are required for full synergistic enhancement of NF-κB-dependent transcription.

To rule out the possibility that the observed effects were due to an impaired binding of p65 to the enzymatic mutants of CARM1 or p300, we performed co-immunoprecipitation assays with nuclear extracts isolated from TNFα stimulated 293T cells expressing either myc-tagged wild-type or enzymatic mutant forms of both, CARM1 or p300, respectively. Both wild-type and enzymatic mutant forms of CARM1 and p300 formed a comparable complex with p65 in the nucleus (Figure 7D left and right panels).

Discussion

The aim of this study was to investigate the role of CARM1, a coactivator for p53- and nuclear receptor-dependent transcription, in NF-κB-dependent gene (Koh *et al*, 2001; An *et al*, 2004). We provide both biochemical and genetic evidence that CARM1 is an essential NF-κB coactivator and a promoter-specific regulator of NF-κB recruitment to chromatin.

Impaired NF-κB-dependent transcription in Carm1-deficient cells

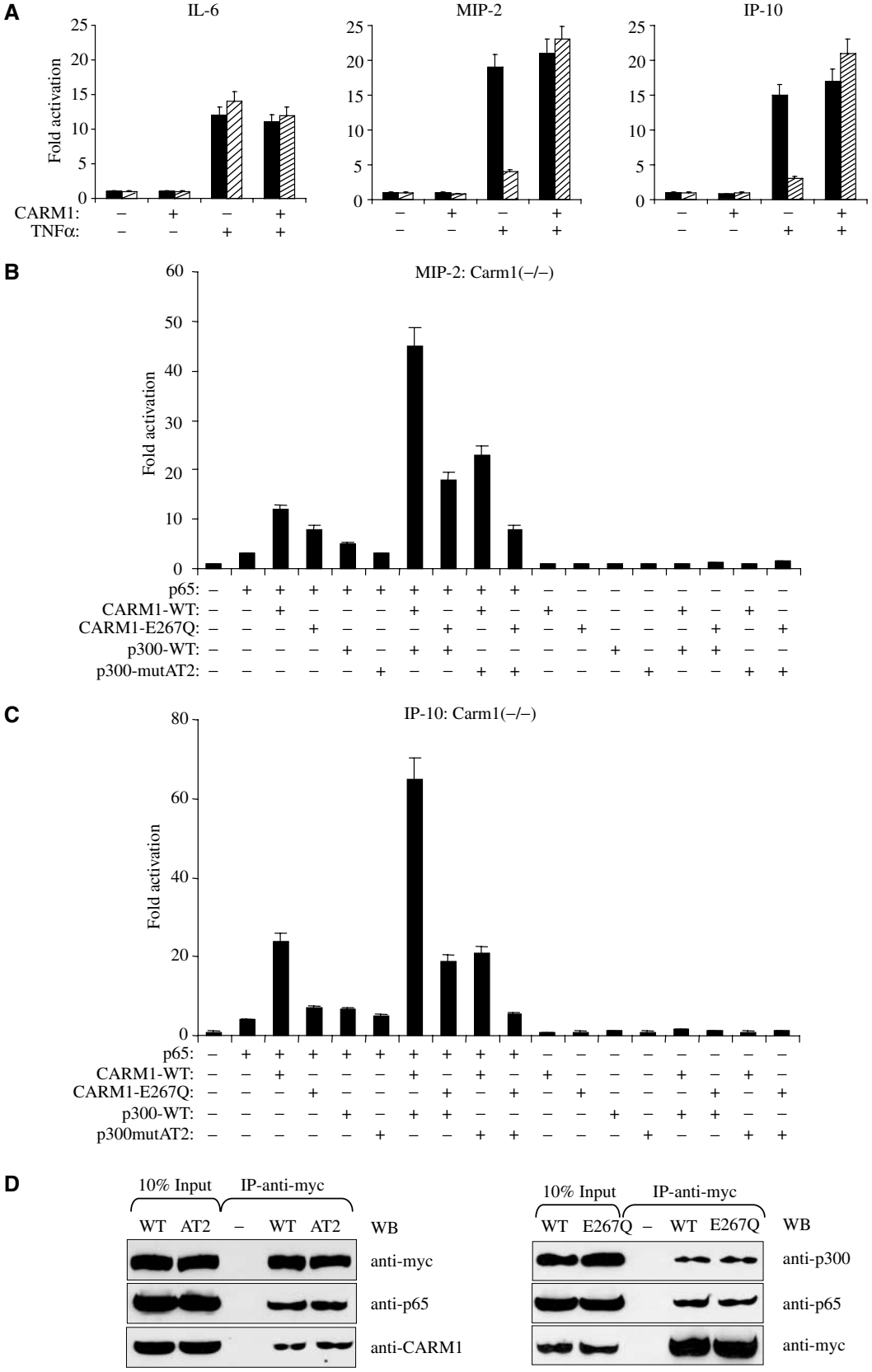
An impaired expression of ICAM-1, G-CSF, MCP-1, IP-10 and MIP-2 was found in TNFα or LPS stimulated Carm1(−/−) fibroblasts. Similarly, NF-κB-dependent reporter gene activity upon LPS or TNFα stimulation was four- to five-fold lower in Carm1(−/−) cells than in wt cells. Interestingly, the expression of IκBα, IL-6, KC and COX-2 was not reduced in Carm1(−/−) fibroblasts upon stimulation, indicating that only a subset of NF-κB-dependent genes is dependent on CARM1. It is unlikely that the canonical pathway of NF-κB transduction cascade is affected by lack of CARM1 since the upstream signaling transducers tested are equally expressed and IκBα degradation and re-synthesis as well as nuclear entry of p65/RelA was similar in Carm1(+/+) and Carm1(−/−) cells. Moreover, the DNA-binding activity of NF-κB(p65/p50) assayed *in vitro* on naked non-chromatinized templates was not impaired in nuclear extracts from Carm1(−/−) fibroblasts, irrespective of the κB site tested.

CARM1 forms a complex with p65 in vivo and in vitro

We could detect endogenous CARM1 in a nuclear complex with p65 and p300, upon TNFα stimulation. Moreover, CARM1 directly binds to the Rel homology domain p65 *in vitro*. The interaction is mediated through the central region

Figure 7 Enzymatic activities of CARM1 and p300/CBP are required for full cooperativity between CARM1 and p300. (**A**) Carm1(+/+) MEF cells (black bars) and Carm1(−/−) MEF cells (dashed bars) were cotransfected with pGL3-MIP-2(−770), pGL3-IP-10(−533) or pOLUC-IL-6(−303/+23) (5 μg) and pphRSVnt-β-gal (200 ng) alone or together with CMV-CARM1 (200 ng) or CMV empty vectors as indicated. Cells were subsequently stimulated with TNFα (10 ng/ml) for 6 h. Cells were harvested 32 h after transfection and the indicated activation of NF-κB-dependent gene expression determined as described in Figure 1. (**B, C**) Enzymatic activities of CARM1 and p300/CBP are required for full cooperativity between CARM1 and p300. Carm1(−/−) MEF cells were cotransfected with pGL3-MIP-2(−770) (B) or pGL3-IP-10(−533) (C) (5 μg) and pphRSVnt-β-gal (200 ng), together with CMV-p65 (80 ng) CMV-CARM1-WT or CARM1-E267Q (200 ng), CMV-p300-WT or p300mutAT2 (750 ng) and CMV empty vectors as indicated. Cells were harvested 32 h after transfection and the indicated activation of NF-κB-dependent gene expression determined as described in Figure 1. (**D**) Both wild type and mutant forms of CARM1 and p300 interact to the same extent with p65 *in vivo*. 293T cells were transfected with CMV expression vectors, either for wild type or enzymatic mutant forms of myc tagged p300 (left panel) or CARM1 (right panel) as well as pphCMV-kozak-myc empty vector. p65, CARM1 and p300 were coimmunoprecipitated (IP) from nuclear extract of TNFα-treated 293T cells (30 min, 10 ng/ml) using an anti-myc antibody. Bound proteins were resolved by SDS–PAGE and subsequently detected by immunoblot (IB) analysis for p65, CARM1 and p300. Input lanes represent 10% of the input.

of CARM1 containing the methyltransferase domain. Since this region is very conserved among members of the PRMT family (Zhang *et al*, 2000), p65 might also interact with other members such as PRMT1. The interaction between full-length p65 and CARM1 was weaker *in vitro*, when compared to the interaction *in vivo*. It is possible that the interaction between



p65 and CARM1 might be modulated or stabilized by post-translational modifications of p65. Whether this modification could be phosphorylation of the Rel homology domain of p65 by protein kinase A (PKA) has yet to be investigated. Phosphorylation of p65 by PKA was shown to open the p65 structure and increase the efficiency of interaction with p300/CBP (Zhong *et al*, 1998). Whether p65, CARM1 and p300 exist in a single complex or different p65/CARM1 and p300/CARM1 complexes form during the transcriptional activation process *in vivo* remains to be elucidated.

Cooperation between CARM1, p300/CBP and p160 family of coactivators in NF-κB-mediated transcription

Depending on the stimulus and the cell type, multiple interactions and the combined actions of distinct transcriptional coactivators seem to be required for the assembly of NF-κB transcription complexes and transcriptional activity of NF-κB. The current study shows that CARM1 synergistically coactivates NF-κB-mediated transactivation, in concert with p300/CBP and the p160 family members. CARM1 was previously shown to function as a secondary coactivator for nuclear receptors (NRs) cooperating with p160 coactivators and p300/CBP under conditions where limiting levels of the NRs are expressed (Lee *et al*, 2002). Similarly, high synergistic enhancement of NF-κB coactivation by CARM1 and protein acetyltransferases was obtained only under conditions where limiting levels of p65 were expressed (data not shown).

Although NRs and NF-κB utilize similar sets of coactivators, there are qualitative differences in the assembly process of each coactivator component by NRs and NF-κB. p160 family members are shown to act as primary coactivators for nuclear receptors and recruit the secondary coactivators CBP/p300 and CARM1 (Lee *et al*, 2002). In contrast, p300/CBP serves as primary coactivator for NF-κB and provides a platform for the secondary coactivators p/CAF and p160 family members (Sheppard *et al*, 1999). Moreover, CARM1 directly interacts with the NF-κB subunit p65 *in vitro* and might act as a primary coactivator for NF-κB.

CARM1 coactivates NF-κB in a promoter-specific manner

At the estrogen-responsive promoter *pS2* and p53-dependent promoter *GADD45*, CARM1 recruitment was shown to follow ER or p53 recruitment, respectively. Thus, CARM1 seems to be recruited by ER or p53 to these promoters (Daujat *et al*, 2002; Metivier *et al*, 2003; An *et al*, 2004). Surprisingly, our ChIP data revealed a major defect in p65 recruitment to the CARM1-dependent MIP-2 and IP-10 promoters in Carm1(−/−) fibroblast upon TNFα stimulation. In contrast, no significant differences could be observed at the IL-6 promoter between Carm1(+/+) and Carm1(−/−) fibroblasts. Since NF-κB dimers from Carm1(−/−) cells retained full DNA-binding activity in EMSA, it can be assumed that one critical role of CARM1 *in vivo* is to promote NF-κB recruitment to selected target genes that, due to their chromatin configuration, are unable to recruit NF-κB 'by default'. This does not rule out the possibility that CARM1 exerts additional essential activities required for NF-κB to activate transcription after it is recruited to chromatin. While H3(R17) methylation at the IL-6 promoter is not important for p65 recruitment, in the case of the IP-10 and MIP-2 promoters the modification of H3 seems to be required. This might be due to a different histone positioning on the IL-6 promoter or due to an 'open' and acessible chromatin conformation maintained by other transcription factors. In contrast, at the IP-10 and MIP-2 promoters, Arg methylation and most likely subsequent modifications of H3 and other histones might enhance κB site accessibility. The specific determinants dictating CARM1 dependency of NF-κB-regulated genes remain to be defined.

The importance of CARM1 enzymatic activity for NF-κB-dependent transcription is supported by complementation experiments in CARM1(−/−) cells using an enzymatic mutant of CARM1 and p300. Full synergistic enhancement of transcription regulated from the MIP-2 and IP-10 promoters was obtained only when wild-type forms of both CARM1 and p300 were coexpressed in CARM1(−/−) cells. Thus, for at least a subset of CARM1-dependent NF-κB target genes, the enzymatic activities of both CARM1 and p300 are necessary for NF-κB-dependent transcription. However, transiently transfected plasmids are thought not to be properly chromatinized, suggesting that nucleosomal histones are unlikely to represent the only relevant substrates whose Arg methylation is required for NF-κB-dependent transcription (Smith and Hager, 1997; Nan *et al*, 2004). Studies regarding the requirement of CARM1-dependent methylation of nonhistone proteins such as p300/CBP (Xu *et al*, 2001; Chevillard-Briet *et al*, 2002) in NF-κB-dependent transcription are ongoing. Since the enzymatic mutant of CARM1 could still partially contribute to NF-κB-dependent transcription, similar to p300/CBP, CARM1 might also act downstream of NF-κB recruitment as a bridging factor. In addition to central methyltransferase/p65-binding domain, the unique N- and C-terminal regions of CARM1 were recently shown to be also required for enhancement of transcriptional activation by nuclear receptors (Teyssier *et al*, 2002). The C-terminal part of CARM1 contains a transactivation domain, which might interact with not yet identified cofactors, thereby driving the equilibrium toward formation of the fully competent NF-κB-dependent coactivator-complex(es).

Interestingly, CARM1 was recruited in a stimulus-dependent manner to all tested promoters, and recruitment correlated with an increase in H3(R17) methylation. Given our results, it seems plausible that CARM1 recruitment to the MIP-2 and IP-10 promoters may be independent of p65 and precede p65 recruitment. It remains to be investigated whether other sequence-specific transcription factors known to cooperate with NF-κB in gene induction, such as STAT1, IRF3 or AP1, are required for CARM1 recruitment to those promoters. The IL-6 promoter reflects a complete different situation, where the TNFα-induced recruitment of CARM1 coincides with p65 recruitment in both Carm1(+/+) and Carm1(−/−) fibroblasts. This could imply that p65 itself might recruit CARM1 to the IL-6 promoter.

It is of great interest to elucidate the relationship of CARM1 to other NF-κB-associated coactivators regarding their relative contribution to NF-κB-dependent gene activation, with respect to different promoters and stimuli. A combination of microarray and genomewide Chip studies using conditional single, double or triple knockout mice or conditional knockin mice expressing enzymatic mutants of particular coactivators could verify their relative contribution and identify the sequential order of particular promoter-specific recruitment cascades *in vivo*.

Materials and methods

Plasmids

Expression vectors for p300 and p65 were described in Hassa *et al* (2003). pphRSV-nt-βgal and the NF-κB-dependent luciferase reporter constructs HIV-luc and HIV-mutκB-luc are described in Hassa *et al* (2001). pGL3-MIP-2(−770), pGL3-IP-10(−533) and pOLUC-IL-6(−303/+23) are described in Han *et al* (1999), Borgland *et al* (2000) and Walpen *et al* (2001). The different CMV-myc expression vectors for CARM1 were obtained by cloning the corresponding PCR products (CARM1 full-length, CARM1—aa 1–148, CARM1—aa 140–480 and CARM1—aa 462–608) in frame into pphCMV-T7-km-3 (Hassa *et al*, 2003). The enzymatic mutant form (E267Q) of CARM1 was created by site-directed mutagenesis as described in Lee *et al* (2002) and verified by sequencing. The CMV-kozak-myc-p300 wild type and p300mutAT2 (enzymatic mutant) were obtained by cloning the corresponding PCR products into pphCMV-T7-km-3. pBKS-p300mutAT2 is described in Kraus *et al* (1999). GST-fusion vectors for p65—aa 1–305 and p65—aa 441–551 were obtained by cloning the corresponding PCR products into pGex6P1.

Cell culture and transient transfection

Carm1(+/+) or Carm1(−/−) MEFs, 293T and COS-1 were grown in DMEM (Invitrogen). Cells were transfected as described previously, except that MEFs were grown for 12 h in DMEM containing 2% fetal calf serum before stimulation with TNFα or LPS (purchased from Sigma). The amount of DNA indicated in the figure legends was calculated for 10 ml of medium. Total amounts of DNA and equal molar ratios of promoters were kept constant in all setups by using empty vectors. For MEFs, only cell passages 2–5 were used for experiments. Owing to differences in transfection efficiencies, an expression plasmid of β-galactosidase (pph-RSV-nt-β-gal) was cotransfected as a transfection efficiency control, and luciferase activities were normalized based on β-galactosidase activity. Luciferase activity was measured as described in Hottiger *et al* (1998).

RNA isolation and RT–PCR analysis

RNA isolation and RT–PCR from Carm1(+/+) or Carm1(−/−) MEFs was performed according to the manufacturer's protocols (Invitrogen). Sequences of primers are available upon request. All PCR products were resolved by 1–2% agarose gel electrophoresis, and DNA bands were visualized by staining the gel with ethidium bromide.

Nuclear extracts, immunoprecipitation and immunoblotting

Nuclear, cytoplasmic and whole-cell extracts were prepared as described in Hottiger *et al* (1998) and Hassa *et al* (2003). All immunoprecipitation and immunoblotting analysis for CARM1, PARP-1, p65, c-Rel, RelB and p300 were performed as described previously (Hottiger *et al*, 1998; Hassa *et al*, 2003). Anti-RelA/p65 (C-20, sc-372), anti-p300 (sc-C260), mouse IgG (sc-2025), rabbit IgG (sc-2027), anti-c-Myc IgG (sc-2027) and anti-α-tubulin (sc-8035) antibodies were obtained from Santa Cruz Biotechnology; anti-PARP-1 antibody (clone C-2–10) was from Anawa trading SA (Switzerland) and anti-p300 antibody (14991A) was from BD PharMingen. The anti-CARM1 antibodies were either described in Kim *et al* (2004) or a generous gift from Dr S Richard (McGill University, Quebec, Canada) and Dr M Stallcup (University of Southern California, USA). The anti-dimethyl-H3-Arg17 antibody (07-214) was from Upstate. The anti-RelB and anti-c-Rel antibodies were kindly provided by Dr NR Rice (National Cancer Institute at Frederick, USA).

In vitro interaction and GST pull-down assay

Recombinant His-mCARM1 and GST-p65 full-length proteins were expressed in Sf21 cells. GST-p65—aa 1–305, -p65—aa 1–441 and -p65—aa 441–551 proteins were expressed in *Escherichia coli* and purified according to the manufacturer's protocols (Novagen, Amersham/Pharmacia Biosciences). All purified proteins were confirmed by Coomassie staining and Western blot analysis using the corresponding antibodies. Coupled *in vitro* transcription/translation reactions were carried out using the TNT T7-Quick system (Promega) according to the manufacturer's protocol. GST pull-down assays were performed in the presence of 120 mM NaCl as described previously (Hassa *et al*, 2003).

ChIP assay

ChIP was carried out as described previously (Saccani and Natoli, 2002). Sequences of promoter-specific primers and a detailed experimental protocol are available upon request.

Acknowledgements

We are grateful to WL Kraus (Cornell University, New York, USA), CK Glass (University of California at San Diego, USA), NR Rice (National Cancer Institute at Frederick, USA), J Pfeilschifter (University of Munster, Germany), AR Brasier (University of Texas Medical Branch, USA), S Richard (McGill University, Quebec, Canada), M Stallcup (University of Southern California, USA) and DA Muruve (University of Calgary, Canada) for the generous provision of reagents. We also thank A Hoffman (University of California at San Diego, USA) for his helpful advice and comments. This work was supported in part by the NIH grant DK62248 and, in part, by institutional grant ES07784 (to MTB), by Swiss National Science Foundation Grant 31-66720.01 (to GN), by Swiss National Science Foundation Grant 31-67771.02 and the Kanton of Zurich (to POH and MOH).

References

Agalioti T, Lomvardas S, Parekh B, Yie J, Maniatis T, Thanos D (2000) Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. *Cell* **103:** 667–678

An W, Kim J, Roeder RG (2004) Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. *Cell* **117:** 735–748

Baeuerle PA, Baltimore D (1988) I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* **242:** 540–546

Baldwin Jr AS (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14:** 649–683

Bauer UM, Daujat S, Nielsen SJ, Nightingale K, Kouzarides T (2002) Methylation at arginine 17 of histone H3 is linked to gene activation. *EMBO Rep* **3:** 39–44

Borgland SL, Bowen GP, Wong NC, Libermann TA, Muruve DA (2000) Adenovirus vector-induced expression of the C-X-C chemokine IP-10 is mediated through capsid-dependent activation of NF-kappaB. *J Virol* **74:** 3941–3947

Chevillard-Briet M, Trouche D, Vandel L (2002) Control of CBP co-activating activity by arginine methylation. *EMBO J* **21:** 5457–5466

Daujat S, Bauer UM, Shah V, Turner B, Berger S, Kouzarides T (2002) Crosstalk between CARM1 methylation and CBP acetylation on histone H3. *Curr Biol* **12:** 2090–2097

Gerritsen ME, Williams AJ, Neish AS, Moore S, Shi Y, Collins T (1997) CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc Natl Acad Sci USA* **94:** 2927–2932

Ghosh S, May MJ, Kopp EB (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16:** 225–260

Goodman RH, Smolik S (2000) CBP/p300 in cell growth, transformation, and development. *Genes Dev* **14:** 1553–1577

Han Y, Runge MS, Brasier AR (1999) Angiotensin II induces interleukin-6 transcription in vascular smooth muscle cells through pleiotropic activation of nuclear factor-kappa B transcription factors. *Circ Res* **84:** 695–703

Hassa PO, Buerki C, Lombardi C, Imhof R, Hottiger MO (2003) Transcriptional coactivation of nuclear factor-kappaB-dependent gene expression by p300 is regulated by poly(ADP)-ribose polymerase-1. *J Biol Chem* **278:** 45145–45153

Hassa PO, Covic M, Hasan S, Imhof R, Hottiger MO (2001) The enzymatic and DNA binding activity of PARP-1 are not required

for NF-kappa B coactivator function. *J Biol Chem* **276:** 45588–45597

Hottiger MO, Felzien LK, Nabel GJ (1998) Modulation of cytokine-induced HIV gene expression by competitive binding of transcription factors to the coactivator p300. *EMBO J* **17:** 3124–3134

Karin M (1998) The NF-kappa B activation pathway: its regulation and role in inflammation and cell survival. *Cancer J Sci Am* **4** (Suppl 1)**:** S92–S99

Karin M, Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **18:** 621–663

Kim J, Lee J, Yadav N, Wu Q, Carter C, Richard S, Richie E, Bedford MT (2004) Loss of CARM1 results in hypomethylation of thymocyte cyclic AMP-regulated phosphoprotein and deregulated early T cell development. *J Biol Chem* **279:** 25339–25344

Koh SS, Chen D, Lee YH, Stallcup MR (2001) Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities. *J Biol Chem* **276:** 1089–1098

Koh SS, Li H, Lee YH, Widelitz RB, Chuong CM, Stallcup MR (2002) Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and beta-catenin with two different classes of DNA-binding transcriptional activators. *J Biol Chem* **277:** 26031–26035

Kraus WL, Manning ET, Kadonaga JT (1999) Biochemical analysis of distinct activation functions in p300 that enhance transcription initiation with chromatin templates. *Mol Cell Biol* **19:** 8123–8135

Kundu TK, Palhan VB, Wang Z, An W, Cole PA, Roeder RG (2000) Activator-dependent transcription from chromatin *in vitro* involving targeted histone acetylation by p300. *Mol Cell* **6:** 551–561

Lee YH, Koh SS, Zhang X, Cheng X, Stallcup MR (2002) Synergy among nuclear receptor coactivators: selective requirement for protein methyltransferase and acetyltransferase activities. *Mol Cell Biol* **22:** 3621–3632

McBride AE, Silver PA (2001) State of the arg: protein methylation at arginine comes of age. *Cell* **106:** 5–8

Merika M, Thanos D (2001) Enhanceosomes. *Curr Opin Genet Dev* **11:** 205–208

Merika M, Williams AJ, Chen G, Collins T, Thanos D (1998) Recruitment of CBP/p300 by the IFN beta enhanceosome is required for synergistic activation of transcription. *Mol Cell* **1:** 277–287

Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F (2003) Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* **115:** 751–763

Miranda TB, Miranda M, Frankel A, Clarke S (2004) PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity. *J Biol Chem* **279:** 22902–22907

Nan X, Hyndman L, Agbi N, Porteous DJ, Boyd AC (2004) Potent stimulation of gene expression by histone deacetylase inhibitors on transiently transfected DNA. *Biochem Biophys Res Commun* **324:** 348–354

Perkins ND, Felzien LK, Betts JC, Leung K, Beach DH, Nabel GJ (1997) Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator. *Science* **275:** 523–527

Saccani S, Natoli G (2002) Dynamic changes in histone H3 Lys 9 methylation occurring at tightly regulated inducible inflammatory genes. *Genes Dev* **16:** 2219–2224

Schiltz RL, Mizzen CA, Vassilev A, Cook RG, Allis CD, Nakatani Y (1999) Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal substrates. *J Biol Chem* **274:** 1189–1192

Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK, Collins T (1999) Transcriptional activation by NF-kappaB requires multiple coactivators. *Mol Cell Biol* **19:** 6367–6378

Smith CL, Hager GL (1997) Transcriptional regulation of mammalian genes *in vivo*. A tale of two templates. *J Biol Chem* **272:** 27493–27496

Teyssier C, Chen D, Stallcup MR (2002) Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. *J Biol Chem* **277:** 46066–46072

Walpen S, Beck KF, Schaefer L, Raslik I, Eberhardt W, Schaefer RM, Pfeilschifter J (2001) Nitric oxide induces MIP-2 transcription in rat renal mesangial cells and in a rat model of glomerulonephritis. *Faseb J* **15:** 571–573

Whiteside ST, Israel A (1997) I kappa B proteins: structure, function and regulation. *Semin Cancer Biol* **8:** 75–82

Xu W, Chen H, Du K, Asahara H, Tini M, Emerson BM, Montminy M, Evans RM (2001) A transcriptional switch mediated by cofactor methylation. *Science* **294:** 2507–2511

Yadav N, Lee J, Kim J, Shen J, Hu MC, Aldaz CM, Bedford MT (2003) Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. *Proc Natl Acad Sci USA* **100:** 6464–6468

Zhang X, Zhou L, Cheng X (2000) Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. *EMBO J* **19:** 3509–3519

Zhong H, Voll RE, Ghosh S (1998) Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol Cell* **1:** 661–671

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 49, pp. 40450–40464, December 9, 2005

Acetylation of Poly(ADP-ribose) Polymerase-1 by p300/CREB-binding Protein Regulates Coactivation of NF-κB-dependent Transcription*[S]

Received for publication, July 12, 2005, and in revised form, September 28, 2005 Published, JBC Papers in Press, October 4, 2005, DOI 10.1074/jbc.M507553200

Paul O. Hassa[‡], Sandra S. Haenni[‡], Christine Buerki[‡], Nadja I. Meier[‡], William S. Lane[§], Heather Owen[‡], Monika Gersbach[‡], Ralph Imhof[‡], and Michael O. Hottiger[‡1]

From the [‡]Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland and [§]Harvard Microchemistry and Proteomics Analysis Facility, Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

Poly(ADP-ribose) polymerase-1 (PARP-1) and nuclear factor κB (NF-κB) have both been demonstrated to play a pathophysiological role in a number of inflammatory disorders. We recently presented evidence that PARP-1 can act as a promoter-specific coactivator of NF-κB *in vivo* independent of its enzymatic activity. PARP-1 directly interacts with p300 and both subunits of NF-κB (p65 and p50) and synergistically coactivates NF-κB-dependent transcription. Here we show that PARP-1 is acetylated *in vivo* at specific lysine residues by p300/CREB-binding protein upon stimulation. Furthermore, acetylation of PARP-1 at these residues is required for the interaction of PARP-1 with p50 and synergistic coactivation of NF-κB by p300 and the Mediator complex in response to inflammatory stimuli. PARP-1 physically interacts with the Mediator. Interestingly, PARP-1 interacts *in vivo* with histone deacetylases (HDACs) 1–3 but not with HDACs 4–6 and might be deacetylated *in vivo* by HDACs 1–3. Thus, acetylation of PARP-1 by p300/CREB-binding protein plays an important regulatory role in NF-κB-dependent gene activation by enhancing its functional interaction with p300 and the Mediator complex.

Nuclear factor κB (NF-κB) is a widely expressed transcription factor of particular importance to the regulation of cells of the immune system (1). NF-κB encompasses a family of inducible transcription factors including RelA/p65, RelB, c-Rel, p50, and p52 (1). These proteins share a conserved 300-amino acid region within their amino termini, designated Rel-homology domain (RHD). This domain is responsible for dimerization, nuclear translocation, DNA binding, and interaction with heterologous transcription factors (1). NF-κB is composed of homo- or heterodimers with a range of DNA binding and activation potentials. The most abundant and best-studied form of NF-κB in cells is a heterodimer consisting of the two subunits, p50 (NF-κB1) and p65 (RelA). NF-κB plays a key role in the regulation of many genes involved in mammalian immune and inflammatory responses, apoptosis, cell proliferation, and differentiation (1, 2). NF-κB has additionally been associated with neurodegenerative processes and cancer (3, 4). In unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor complex by its physical association with one of several inhibitors of NF-κB (IκBs)[2] (5). Treatment of cells with extracellular stimuli including cytokines, bacterial lipopolysaccharides (LPS), phorbol esters, or potent oxidants leads to rapid phosphorylation of IκBα, which results in ubiquitination of IκBα and subsequent degradation by the 26 S proteasome (4, 5). Dissociation of NF-κB unmasks the nuclear localization sequences of p65 and p50 subunits, which leads to nuclear translocation and binding of NF-κB to specific κB consensus sequences in the chromatin and activation of specific subsets of genes (3).

NF-κB-dependent gene expression requires growing families of transcriptional coactivators (6, 7). The two key coactivators of NF-κB, histone acetyltransferases p300 and its homolog, the cAMP-response element-binding protein (CREB)-binding protein (CBP), directly associate with the NF-κB subunits p50 and p65 (8–10). These coactivators are thought to promote the rapid formation of the pre-initiation and re-initiation complexes by bridging the sequence-specific activators (like NF-κB) to the basal transcription machinery, thereby facilitating multiple rounds of transcription (11). Additionally, the histone acetyltransferases p300 and CBP can modify the amino-terminal tails of nucleosomal histones, thereby altering the local chromatin structure (12–14). Although the recruitment of p300 or CBP to NF-κB-dependent enhanceosomes is required for synergistic activation, tethering p300/CBP alone to the promoter through NF-κB is not sufficient for full activity of NF-κB in the context of chromatin. Several reports indicated that the combined actions and interactions of distinct transcriptional coactivator complexes and cofactors seem to be attributable to the strong transcriptional activity of NF-κB, depending on the stimuli and the cell type (6, 8, 15, 16).

Poly(ADP-ribose) poly-merase-1 (PARP-1) is a nuclear chromatin-associated protein and belongs to a large family of enzymes that can synthesize polymers of ADP-ribose units by using β-nicotinamide adenine dinucleotide (NAD^+) as substrate (17). PARP-1 and NF-κB have both been suggested to play a pathophysiological role in a number of inflammatory disorders (17). Several studies showed that PARP-1(−/−) mice were protected against myocardial infarction, streptozotocin-induced diabetes, LPS-induced septic shock, zymosan-induced vascular

* This work was supported in part by the Bonnizzi-Theler and Velux Foundation and the Swiss National Science Foundation Grant 31-67771.02. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental material.

[1] Supported by the Kanton of Zurich. To whom correspondence should be addressed: Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich. Tel.: 41-44-635-54-74; Fax: 41-44-635-68-40; E-mail: hottiger@vetbio.unizh.ch.

[2] The abbreviations used are: IκB, inhibitor of NF-κB; CBP, cAMP-response element-binding protein (CREB)-binding protein; PCAF, p300/CBP-associated factor; AcK, lysin-acetylation domain; LPS, lipopolysaccharide; PARP-1, poly(ADP-ribose) polymerase-1; TNF, tumor necrosis factor; IFN, interferon; iNOS, inducible nitric oxide synthase; HDAC, histone deacetylase; GST, glutathione *S*-transferase; CMV, cytomegalovirus; CDK, cyclin-dependent kinase; NAM, nicotinamide; siRNA, small interfering RNA; USA, upstream stimulatory activity; MIP-2, macrophage inflammatory protein-2; TSA, trichostatin A; PCAF, p300/CBP-associated factor; HMG, high mobility group; KC, keratinocyte-derived cytokine.

failure, a non-septic model of multiple organ dysfunction as well as collagen-induced arthritis. This indicates that PARP-1 has a vital role in inflammatory disorders (17). PARP-1 has been suggested to act as a promoter-specific coactivator of NF-κB in these inflammatory disorders (18). Indeed, up-regulation of subsets of inflammatory mediators such as TNFα, IFNγ, and iNOS was shown to be impaired in PARP-1(−/−) mice upon treatment with LPS, zymosan, or streptozotocin (17, 19). We recently presented evidence that PARP-1 can act as a coactivator of NF-κB *in vivo* (18). Neither the nuclear translocation nor the DNA binding ability of NF-κB was affected in PARP-1(−/−) cells (18). However, PARP-1 directly interacted with both subunits of NF-κB (p65 and p50) *in vitro* and *in vivo* (18, 20). Remarkably, the enzymatic activity of PARP-1 was not required for full activation of NF-κB in response to various stimuli *in vivo* (20). In addition, PARP-1 directly interacted with p300 and synergistically coactivated NF-κB-dependent transcription (8). Tulin and Spradling (21) recently found that *Drosophila* mutants lacking normal PARP-1 levels display immune defects similar to mice lacking the NF-κB subunit p50. Their results imply that the role of PARP-1 in NF-κB-dependent gene expression during immune responses has been conserved during evolution.

Because NF-κB-dependent gene expression requires post-translational modifications (22), we decided to test whether the coactivator activity of PARP-1 might also be regulated by post-translational modifications. We show here that PARP-1-dependent gene expression not only requires the enzymatic activity of p300/CBP but also that PARP-1 itself is acetylated *in vivo* in response to inflammatory stimuli. Acetylation sites were mapped *in vitro* and *in vivo* to Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524. Furthermore, acetylation of PARP-1 at these lysines is required for the interaction with p50 and the transcriptional activation of NF-κB in response to inflammatory stimuli. Interestingly, the synergistic coactivation of PARP-1, p300, and also the Mediator complex was dependent on acetylation of PARP-1. Moreover, PARP-1 interacted with and is potentially deacetylated *in vivo* by class I HDACs. PARP-1-dependent transcriptional activation of NF-κB was negatively regulated by HDACs 1–3 *in vivo*. Together, these results support the hypothesis that acetylation of PARP-1 is important for its role as transcriptional coactivator and that the different physiological functions of PARP-1 might be in general regulated by post-translational modifications in a stimulus-dependent manner.

MATERIALS AND METHODS

Reagents—Recombinant TNFα was obtained from R&D Systems. Phorbol esters and mouse interferon-γ was purchased from Sigma. LPS (*Escherichia coli*, O26:B6) prepared by phenol extraction was purchased from Sigma and prepared as dispersed sonicate in endotoxin-free water before diluting to a final concentration in supplemented media. Nonfat dry milk was obtained from Migros (Switzerland). Nitrocellulose membranes were purchased from Osmonics Inc. Tosyl-activated Dynabeads were purchased from Dynal Biotech GmbH.

Plasmids—GST-PARP-1 and GST-p50 full-length expression vectors, CMV-PARP-1, and CMV-p300 expression vectors were described in Hassa *et al.* (8) and Covic *et al.* (16). The CMV expression vectors for the different Mediator subunits were generated by PCR or were a generous gift from Dr. L. Freedman (Merck Research Laboratories, West Point, NY). The CMV expression vectors for Myc-tagged HDACs 1–3 were created by PCR. The CMV expression vectors for FLAG or hemagglutinin-tagged HDAC 4–6 were kind gifts from Dr. S. L. Schreiber (Harvard University, Cambridge, MA). The expression vectors for CDK8/cyclin C and subunits of TFIID and TFIIF were kind gifts from Dr. M. Otsuka (University of Tokyo, Japan), Dr. Z. F. Burton (Michigan State University, East Lansing, MI), and Dr. G. Napolitano (University of Naples "Frederico II," Naples, Italy). NF-κB-dependent luciferase reporter constructs for MIP-2 (MIP-2 (−531/wt and MIP-2 (-531/mutκB)-Luc) and iNOS (iNOS (1485/+31wt)-Luc and iNOS(1485/+31-mutκB)-Luc) were generous gifts from Dr. H.-J. Kwon, (Yonsei University, Seoul, Korea) and Dr. M. A. Perrella (Harvard School of Public Health, Boston, MA) and are described in Perrella *et al.* (23) and Kim *et al.* (24). pphRSV-nt-β-galactosidase is described in Hassa *et al.* (20). The baculovirus for CDK8 and cyclin C was a generous gift from Dr. R. Pinhero (University of Guelph, Ontario, Canada) (25). The baculovirus for human p300 and mouse CBP was a generous gift from Dr. W. L. Kraus (Cornell University, New York) and Dr. D. Thanos (Institute of Molecular Biology and Genetics, Alexander Fleming Biomedical Sciences Research Center, Athens, Greece). The baculovirus for PARP-1 full-length and different domains were created by PCR using the PAK-8/9 system (BD Biosciences). GST-fusion expression vectors for different domains of PARP-1 were created by PCR. The PARP-1 mutant constructs were obtained by site-directed mutagenesis and confirmed by sequencing.

Mice Breeding Conditions, Cell Culture, and Transient Transfections—129S/EV-PARP-1(+/+) and 129S/EV-PARP-1(−/−) mice were bred under specified pathogen-free conditions. The initial 129S/EV-PARP-1(+/+) and 129S/EV-PARP-1(−/−) mice breeding pairs were a kind gift from Dr. Z. Q. Wang (26) (International Agency for Research on Cancer, Lyon, France). Primary macrophage cells were isolated from fresh littermates of 129S/EV-PARP-1(+/+) and 129S/EV-PARP-1(−/−) mice (20, 26) according to an isolation procedure described in Petrilli *et al.* (19), and human monocyte/macrophage-like THP-1 cells and Jurkat-T cells were grown in Hepes-buffered RPMI-Glutamax-I (Invitrogen) containing 10% fetal calf serum U. S.-certified (Invitrogen) and supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, and minimum Eagle's medium-nonessential amino acids. Primary mouse fibroblast cells were grown in Hepes-buffered Dulbecco's modified Eagle's medium-Glutamax-I (Invitrogen) containing 4.5 g/liter glucose, 10% fetal calf serum U. S.-certified (Invitrogen) and supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, and minimum Eagle's medium-nonessential amino acids. HEK293 cells were grown in Hepes-buffered Dulbecco's modified Eagle's medium-Glutamax-I (Invitrogen) containing 4.5 g/liter glucose and 10% fetal calf serum U. S.-certified (Invitrogen) and supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen), and minimum Eagle's medium-nonessential amino acids. For luciferase reporter assays, cells were grown in 24- or 48-well dishes. Cells were transfected using calcium phosphate or polyethyleneimine procedures (as described in Refs. 27–30) except that primary cells were grown for 12 h in RPMI medium containing 2% fetal calf serum before stimulation with TNFα or LPS/IFNγ. The amount of DNA indicated in the figure legends was calculated for 10 ml of medium. Total amounts of DNA and equal molar ratios of promoters were kept constant in all set-ups by using empty vectors. For primary cells only cell passages 1–4 were used for transfection experiments. Because of differences in transfection efficiencies, an expression plasmid of β-galactosidase (pph-RSV-nt-β-Gal) was co-transfected as a transfection efficiency control, and luciferase activities were normalized based on β-galactosidase activity. Luciferase activity was measured as previously described in Hottiger *et al.* (27). For further experimental procedures see the supplemental material.

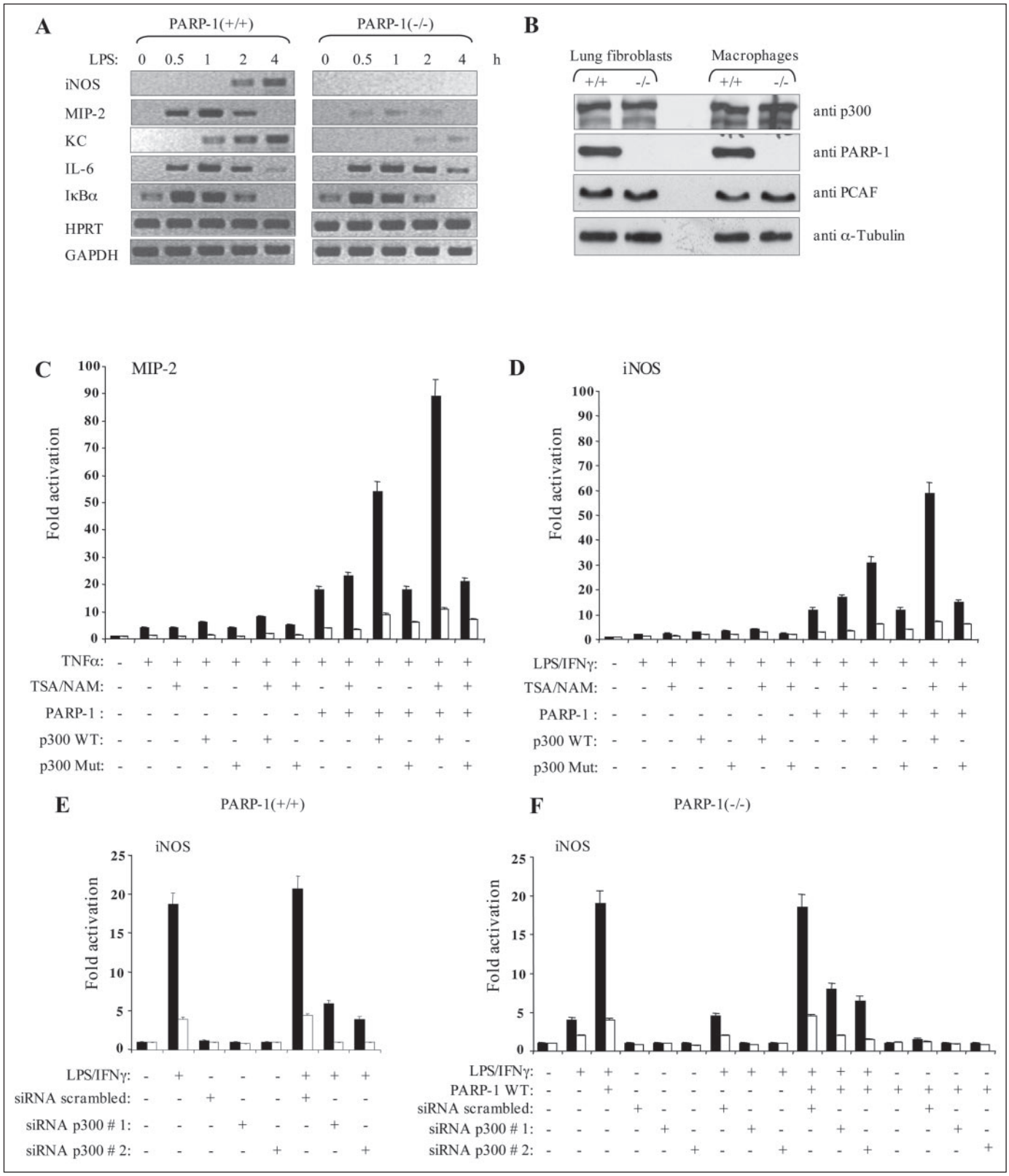


FIGURE 1. **PARP-1 is acetylated upon stimulation *in vivo* and requires the enzymatic activity of p300 for full NF-κB-dependent transcriptional activity.** *A*, primary PARP-1(+/+) or PARP-1(−/−) macrophages were treated with LPS (0.1 μg/ml) as indicated, and the expression of hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and NF-κB-dependent genes KC, MIP-2, iNOS, IκBα and interleukin-6 (*IL-6*) was assessed by reverse transcription-PCR. *B*, equal amounts of total cell extracts from primary PARP-1(+/+) and PARP-1(−/−) lung fibroblasts or primary PARP-1(+/+) and PARP-1(−/−) macrophages were resolved by SDS-PAGE followed by immunoblot analysis using anti-p300, anti-PARP-1, anti-PCAF, or anti- α-tubulin antibodies. *C* and *D*, primary PARP-1(−/−) macrophages were cotransfected with RSV-nt-β-Gal (300 ng), expression vectors for PARP-1 (2 μg), and wild type (*WT*) or an enzymatic mutant (*Mut*) of p300 (2 μg) along with a luciferase reporter under the control of the endogenous MIP-2 (1 μg) or iNOS (3 μg) promoters; cells were subsequently treated for 4 h with TNFα (10 ng/ml) or LPS/IFNγ (0.05 μg/ml/100 units) in the simultaneous presence or absence of low doses of deacetylase inhibitors (10 nM TSA/200 μM NAM). Cells were harvested 24 h after transfection, and NF-κB-dependent gene expression was determined. The indicated activation was determined by the ratio of the relative luciferase activity measured for the promoters containing wild type κB sites (*black bars*) or mutκB sites (*white bars*) after stimulation. The ratio

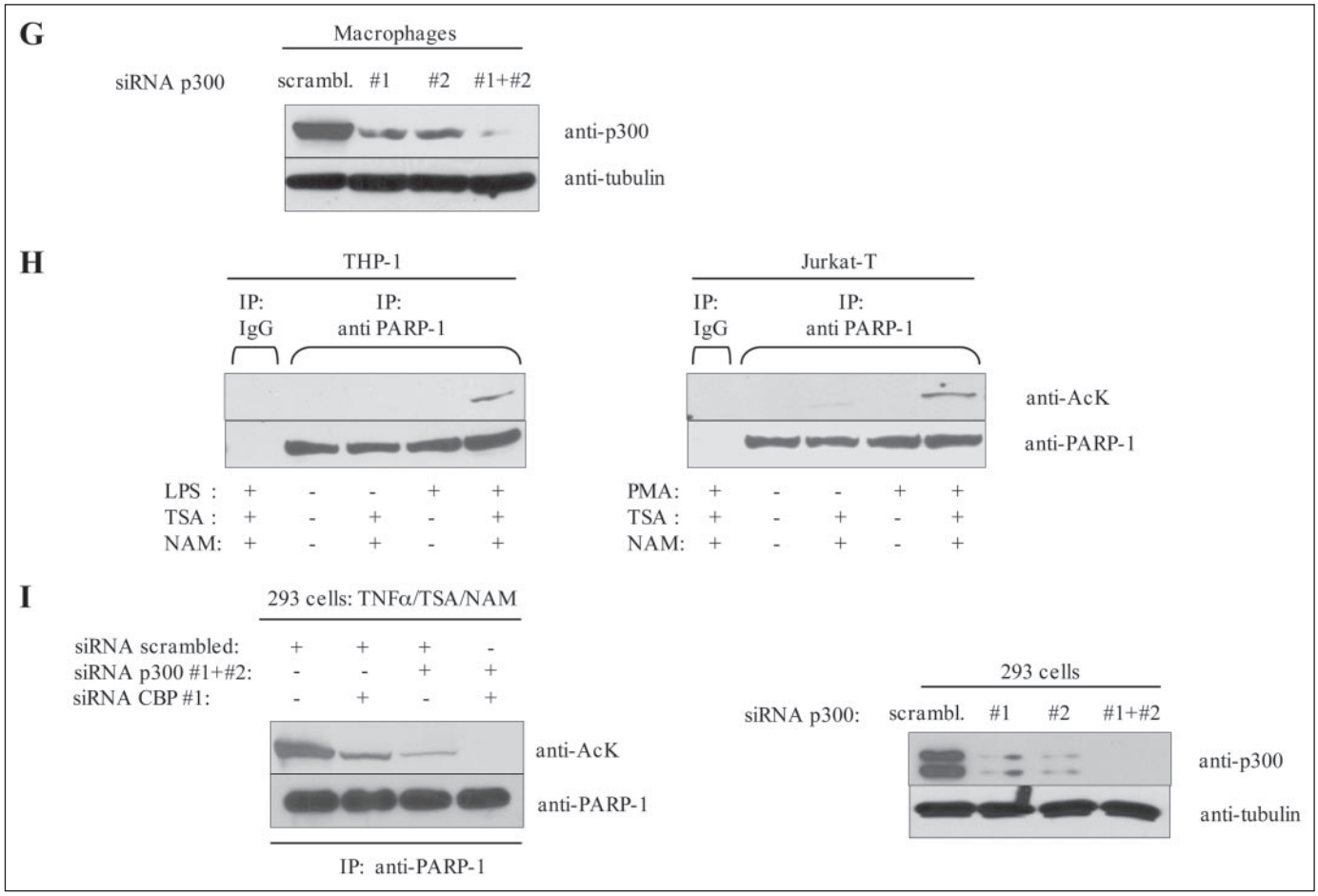


FIGURE 1—*continued*

RESULTS

PARP-1 Requires the Enzymatic Activity of p300 for Full NF-κB-dependent Transcriptional Activity and Is Acetylated in Vivo by p300/CBP upon Stimulation—Because the coactivator activity of PARP-1 for NF-κB-dependent gene expression is dependent on the stimuli and cell type (17), we first tested which NF-κB-dependent genes are impaired in freshly isolated primary PARP-1(−/−) macrophages. Primary PARP-1(+/+) or PARP-1(−/−) macrophages were treated with LPS as indicated, and the expression of NF-κB-dependent genes was assessed by reverse transcription-PCR (Fig. 1*A*). The experiments revealed that LPS-induced levels of KC, MIP-2, and iNOS were impaired in PARP-1(−/−) cells (Fig. 1*A*). The expression of IκBα and interleukin-6 (*IL-6*) was not reduced (Fig. 1*A*), indicating that only a subset of NF-κB-dependent genes requires PARP-1 for gene induction in these cells. Similar results were obtained when cells were stimulated with TNFα (data not shown). To confirm that the expression levels of histone acetyltransferases are not reduced in primary PARP-1 (−/−) cells, we tested the protein levels of p300 and PCAF by immunoblot analysis using anti-PARP-1, anti-p300, or anti-PCAF antibodies. The endogenous protein levels of p300 and PCAF were not impaired in freshly isolated primary PARP-1(−/−) lung fibroblast or macrophage cells (Fig. 1*B*).

PARP-1 and p300/CBP were shown to form a complex and function synergistically to enhance NF-κB-mediated gene expression (8). To test whether the synergistic coactivation of NF-κB-mediated transactivation by PARP-1 and p300/CBP might require the enzymatic activity of p300/CBP, we transfected PARP-1(−/−) cells with expression vectors for PARP-1 and wild type or an enzymatic mutant of p300 along with a luciferase reporter under the control of the endogenous MIP-2 or iNOS promoters, shown to be PARP-1-dependent. Cells were subsequently treated with TNFα or LPS/IFNγ in the simultaneous presence or absence of low doses of deacetylase inhibitors (TSA and nicotinamide (NAM)) (Fig. 1, *C* and *D*). Coexpression of wild type p300 with PARP-1 in PARP-1(−/−) cells resulted in a highly synergistic enhancement of transcription regulated from both MIP-2 and iNOS promoters upon stimulation (Fig. 1, *C* and *D*). However, the cooperativity between p300 and PARP-1 was severely impaired when an enzymatic mutant of p300

obtained for untreated cells was arbitrarily set to 1. *Error bars* indicate S.E. of three independent experiments. *E* and *F*, primary PARP-1(+/+) and PARP-1(−/−) macrophages were repeatedly transfected with scrambled- or p300-siRNA targeting vector DNA (30 μg) as indicated and then transfected and treated as indicated and described in *D*. The indicated activation was determined as described in *D*. *G*, equal amounts of total cell extracts from primary macrophages repeatedly transfected with scrambled- or p300-siRNA targeting vector DNA (30 μg) as indicated were resolved by SDS-PAGE followed by immunoblot analysis using anti-p300 or anti- α-tubulin antibodies. *H*, THP-1 or Jurkat-T cells were treated as indicated with LPS (0.2 μg/ml) or phorbol ester (10 nM) and simultaneously with or without deacetylase inhibitors (100 nM TSA/400 μM NAM) for 30 min. PARP-1 was immunoprecipitated (*IP*) under high salt conditions from nuclear extracts, and the presence of acetylated forms of PARP-1 was subsequently tested by immunoblot analysis using anti-AcK or anti-PARP-1 antibodies. *PMA*, phorbol 12-myristate 13-acetate. *I*, *left panel*, 293 cells were repeatedly transfected with scrambled-, p300-, or CBP-siRNA targeting vector DNA (30 μg) and treated as indicated. PARP-1 was immunoprecipitated under high salt conditions from nuclear extracts, and the presence of acetylated forms of PARP-1 was subsequently tested by immunoblot analysis using anti-AcK or anti-PARP-1 antibodies. *Right panel*, equal amounts of total cell extracts from 293 cells repeatedly transfected with scrambled- or p300-siRNA targeting vector DNA (30 μg) as indicated were resolved by SDS-PAGE followed by immunoblot analysis using anti-p300 or anti- α-tubulin antibodies.

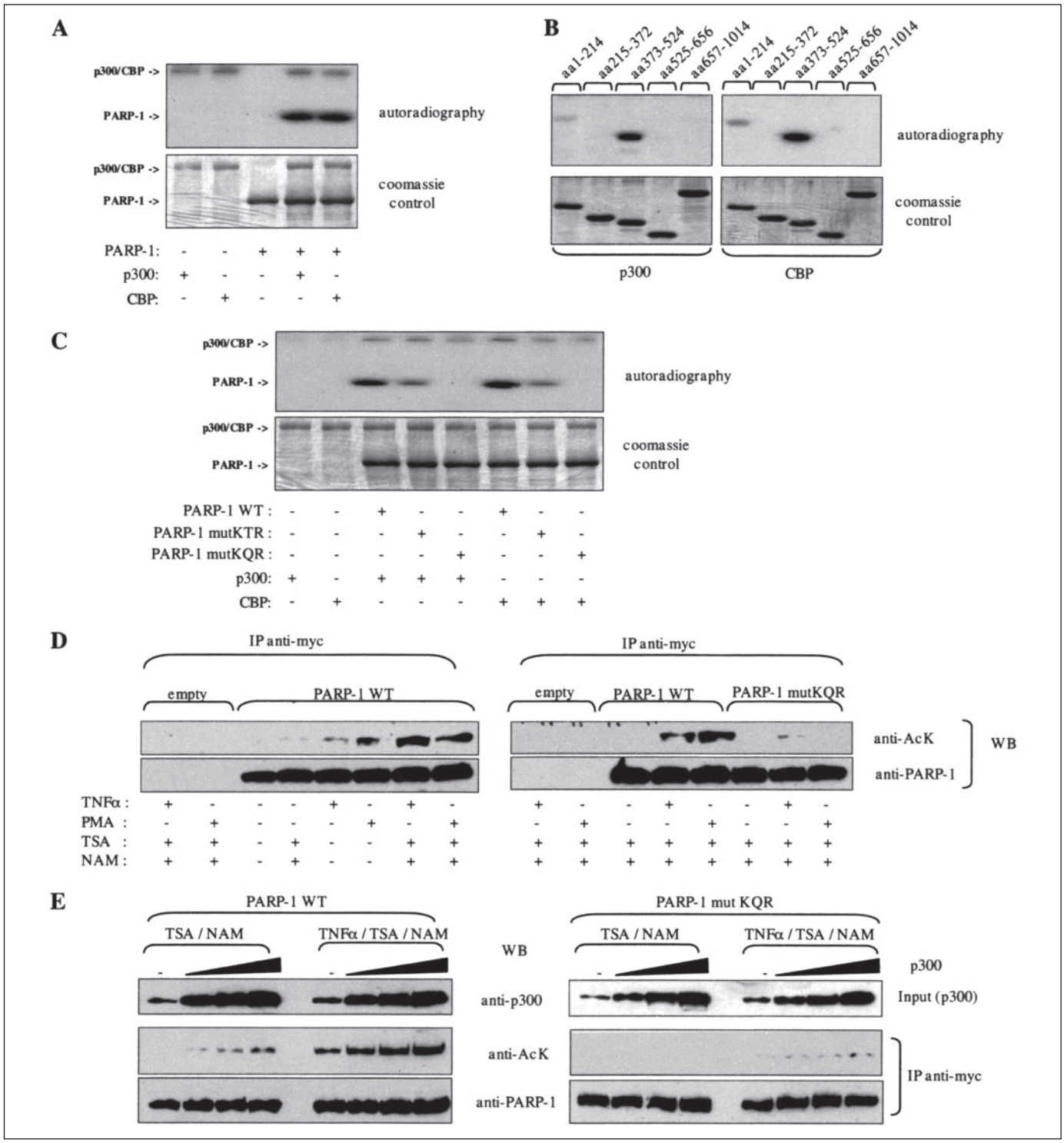


FIGURE 2. **PARP-1 is acetylated *in vitro* and *in vivo* at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 by p300/CBP upon stimulation.** *A*, recombinant purified full-length PARP-1 was incubated with recombinant purified full-length p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography. *B*, baculo-purified PARP-1 fragments corresponding to amino acids (*aa*) 1–214, 215–372, 373–525, 525–656, or 657–1014 were incubated with recombinant purified full-length p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography. *C*, recombinant purified full-length PARP-1 wild type and two mutant forms of PARP-1; mutKTR (K498R/K521R/K524R) and mutKQR (K498R/K505R/K508R/K521R/K524R) were incubated with recombinant purified full-length p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography. *D*, Myc-tagged PARP-1 wild type (*WT*) or mutant mutKQR were overexpressed in 293 cells and treated as indicated with TNFα (10 ng/ml) and/or deacetylase inhibitors (100 nM TSA/400 μM NAM) for 30 min. Myc-tagged PARP-1 wild type or mutant mutKQR was then immunoprecipitated (*IP*) under high salt conditions from nuclear extracts of 293 cells, and the presence of acetylated forms of PARP-1 was subsequently tested by immunoblot analysis (*IB*) using anti-AcK or anti-PARP-1 antibodies. *E*, the same experiments were repeated in the presence of increasing amounts of overexpressed p300. Immunoblot of p300 input is shown in the *upper panels*

was coexpressed. The most striking differences between wild type and the enzymatic mutant of p300 were observed in the presence of low doses of deacetylase inhibitors (Fig. 1, *C* and *D*). The same transfection experiments with a reporter gene under the control of mutated κB sites revealed that the observed cooperative effect of p300 and PARP-1 was mainly NF-κB-specific (Fig. 1, *C* and *D*). To further confirm these

results, the same experiments were repeated in the presence of different p300-siRNA targeting vectors (Fig. 1, *E* and *F*). These experiments revealed that the presence of endogenous p300 is required for PARP-1-dependent coactivation of NF-κB-dependent gene expression (Fig. 1, *E* and *F*). Together these results indicate that the enzymatic activity of p300/CBP is required for NF-κB-dependent transactivation of extrachromosomal templates upon treatment with inflammatory stimuli.

p300 and CBP are known to modify a variety of proteins, such as histones and transcription factors (31). To investigate whether endogenous PARP-1 itself might be acetylated by p300/CBP *in vivo*, we immunoprecipitated endogenous PARP-1 under high salt conditions from nuclear extracts of THP-1 or Jurkat-T cells upon treatment with the indicated stimuli (LPS or TNFα) in the simultaneous presence or absence of deacetylase inhibitors (Fig. 1*H*, *left* and *right panels*). Possible acetylation of PARP-1 was analyzed by immunoblot analysis using an anti-AcK antibody. Endogenous PARP-1 was acetylated *in vivo* only in the presence of deacetylase inhibitors upon stimulation of these cells (Fig. 1*H*, *left* and *right panels*). Next we tested whether the observed acetylation of PARP-1 is dependent on p300/CBP. 293 cells were cotransfected with a control-siRNA vector and/or different siRNA targeting vectors for p300 or CBP. Endogenous PARP-1 was immunoprecipitated under high salt conditions from nuclear extracts of 293 cells upon treatment with TNFα in the presence of deacetylase inhibitors (Fig. 1*H*, *left* and *right panels*). These results revealed that endogenous PARP-1 was only acetylated *in vivo* upon stimulation in presence of endogenous p300 or CBP (Fig. 1*I*).

PARP-1 Is Acetylated by p300/CBP in Vitro and in Vivo at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524—Next, we tested whether PARP-1 can be acetylated by p300 or CBP *in vitro*. Recombinant-purified full-length PARP-1 was incubated with recombinant-purified full-length p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography analysis (Fig. 2*A*). PARP-1 was strongly acetylated *in vitro* by p300 or CBP (Fig. 2*A*). To map the domains within PARP-1 subjected to acetylation by p300 and CBP, the same *in vitro* acetylation assay was repeated with different PARP-1 fragments corresponding to amino acids 1–214, 215–372, 373–525, 525–656, or 657–1014. These experiments revealed that p300 and CBP strongly acetylated the PARP-1 domain corresponding to amino acids 373–525 (Fig. 2*B*, *left* and *right panels*). Interestingly, a longer autoradiography exposure revealed that the domain corresponding to amino acids 1–214 of PARP-1 and to the small product of caspase cleaved PARP-1, was weakly acetylated by p300 or CBP (supplemental information and data not shown). The physiological relevance of this finding is currently under investigation. To identify the lysines acetylated by p300/CBP within PARP-1, recombinant purified full-length PARP-1 was acetylated *in vitro* by p300 full-length and analyzed by microcapillary reverse-phase high performance liquid chromatography nanoelectrospray tandem mass spectrometry. Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 were identified as strong candidates for acetylation. To confirm that these lysines are the main acceptor sites, full-length PARP-1 wild type or two PARP-1 mutant forms, mutKTR (K498R/K521R/K524R) and mutKQR (K498R/K505R/K508R/K521R/K524R) were incubated with p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography analysis (Fig. 2*C*). These experiments confirmed that only the PARP-1 mutant harboring all five mutated lysines was no longer acetylated *in vitro* by p300 and CBP (Fig. 2*C*). To investigate whether these lysines are also the main acceptor sites for acetylation *in vivo* by p300, we immunoprecipitated Myc-tagged PARP-1 wild type or mutant mutKQR under high salt conditions from nuclear extracts of 293 cells upon treatment with the indicated stimuli in the presence or absence of deacetylase inhibitors (Fig. 2*D*). The presence of acetylated forms of PARP-1 was tested by immunoblot analysis using an anti-AcK antibody. PARP-1 was acetylated *in vivo* at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 in the presence of deacetylase inhibitors upon stimulation (Fig. 2*D*, also see the supplemental information). The same experiments performed in the presence of increasing amounts of overexpressed p300 suggested that these lysines are acetylated *in vivo* by p300 (Fig. 2*E*).

Acetylation of PARP-1 at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 Is Required in Vivo for Full NF-κB-dependent Transcriptional Activity—To directly test whether acetylation of PARP-1 is required for NF-κB-dependent transcriptional activity *in vivo*, we transfected PARP-1(−/−) cells with expression vectors for p300 and PARP-1 wild type or mutant mutKQR along with a luciferase reporter under the control of the endogenous MIP-2 or iNOS promoters. Cells were subsequently treated with the indicated stimuli (TNFα or LPS/IFNγ) in the simultaneous presence or absence of low doses of deacetylase inhibitors (Fig. 3, *A* and *B*). Coexpression of wild type p300 and wild type PARP-1 in PARP-1(−/−) cells caused a highly synergistic enhancement of transcription regulated from both MIP-2 and iNOS promoters upon stimulation (Fig. 3, *A* and *B*). However, the cooperativity between p300 and PARP-1 was severely impaired when the PARP-1 mutant mutKQR was coexpressed. The most striking differences between PARP-1 wild type and the mutant mutKQR were observed in the presence of low doses of deacetylase inhibitors. The same transfection experiments with a reporter gene under the control of mutated κB sites revealed that the observed induction was mainly NF-κB-specific (Fig. 3, *A* and *B*; and supplemental information).

Pavri *et al.* (32) have very recently shown that PARP-1 can associate *in vivo* with the Mediator. Therefore, we repeated the same transfection experiments in the presence of overexpressed subunits of the Mediator complex. Coexpression of PARP-1 wild type, p300, and Mediator subunits in PARP-1(−/−) cells caused a synergistic enhancement of NF-κB-dependent transcription, whereas no synergistic enhancement was observed when the mutant form of PARP-1, mutKQR, was coexpressed (Fig. 3, *C* and *D*), indicating that acetylation of PARP-1 is also required for the transcriptional cooperativity between p300/CBP, Mediator, and PARP-1 on these promoters.

PARP-1 Interacts in Vivo with the Mediator Complex and in Vitro Directly with the Mediator Subunits CDK8 and DRIP150—To further confirm these data and to investigate whether CDK8 or other subunits of the Mediator complex might directly interact with PARP-1, we first coexpressed tagged forms of different Mediator subunits in 293 cells (Fig. 4*A*). Immunoprecipitation experiments using an anti-PARP-1 antibody revealed that PARP-1 interacted with the whole Mediator complex (Fig. 4*A*). Next, we repeated these experiments with primary macrophages (Fig. 4*B*). These experiments revealed that endogenous PARP-1 could indeed interact with the endogenous Mediator complex under physiological conditions (Fig. 4*B*). DNA did not mediate the association of PARP-1 with the Mediator in the nucleus since the presence of ethidium bromide or DNase I did not affect PARP-1/Mediator interaction (data not shown). Surprisingly, whereas the interaction of PARP-1 with the core Mediator module was increased upon stimulation, the interaction of PARP-1 with CDK8 was decreased (Fig. 4, *A* and *B*). CDK8 is thought to act mainly as a repressor sub-module of the Mediator complex (32).

Because these results strongly suggested that PARP-1 would directly interact with at least one of these Mediator subunits, recombinant purified GST-PARP-1 full-length was bound to glutathione beads followed by incubation with *in vitro* translated and radioactive-labeled Mediator

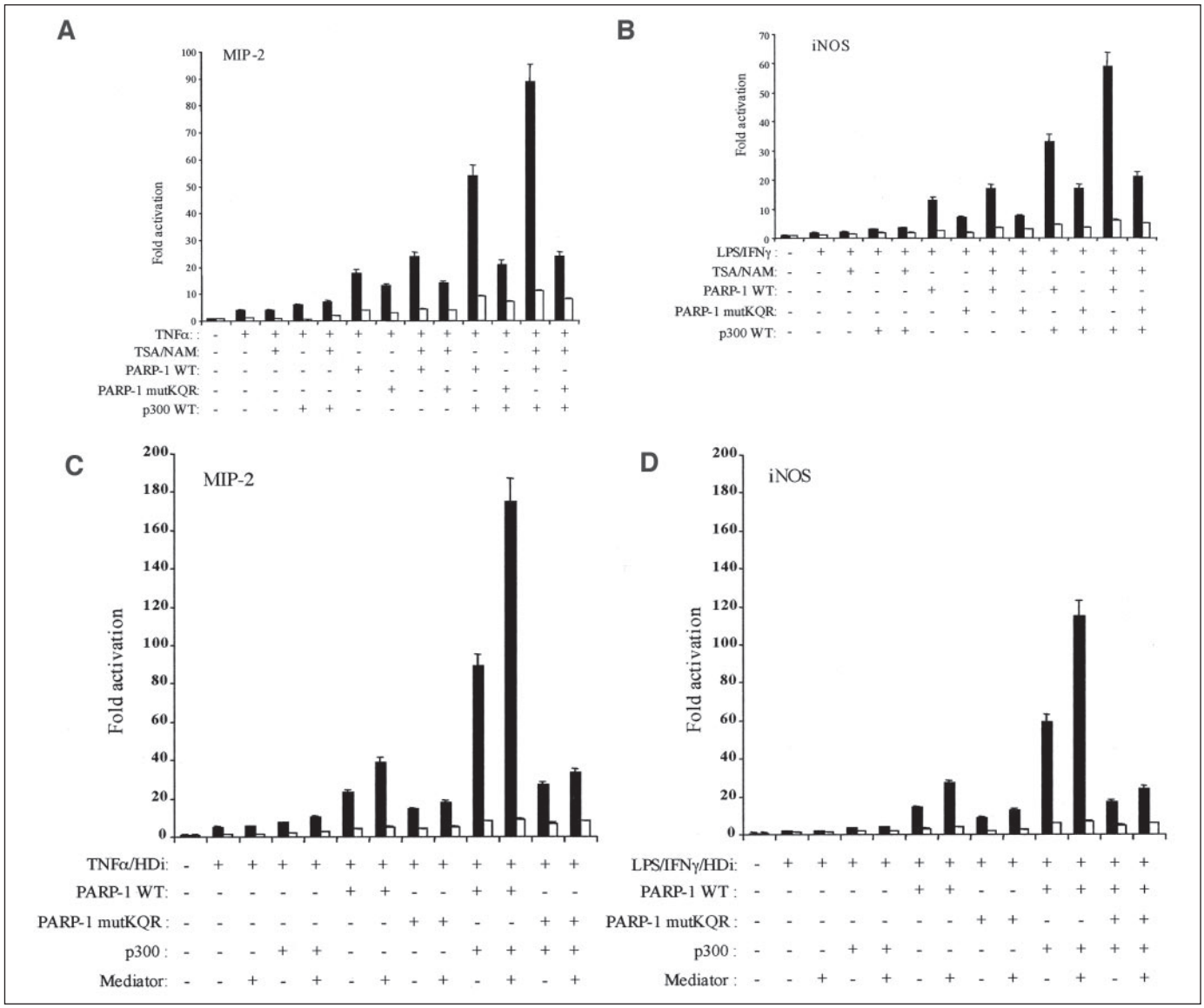


FIGURE 3. **Acetylation of PARP-1 is required for NF-κB-dependent transcription and for the transcriptional cooperativity between PARP-1, p300/CBP, and Mediator *in vivo*.** *A* and *B*, primary PARP-1(−/−) macrophages were cotransfected with RSV-nt-β-Gal (300 ng), expression vectors for p300 (2 μg), and PARP-1 wild type (*WT*) or mutant mutKQR (2 μg) along with a luciferase reporter under the control of the endogenous MIP-2 (1 μg) (*A*) or iNOS (3 μg) (*B*) promoters; cells were subsequently treated for 4 h with TNFα (10 ng/ml) or LPS/IFNγ (0.05 μg/ml/100 units) in the simultaneous presence or absence of low doses of deacetylase inhibitors (10 nM TSA/200 μM NAM). Cells were harvested 24 h after transfection, and NF-κB-dependent gene expression determined as described in Fig. 1*C*. *C* and *D*, primary PARP-1(−/−) macrophages were cotransfected with RSV-nt-β-Gal (300 ng), expression vectors for p300 (2 μg), MED-1 (2.5 μg), MED-7 (0.4 μg), MED-14 (2 μg), MED-15 (1.1 μg), MED-17 (0.75 μg), MED-23 (1.5 μg), MED-24 (1 μg), and PARP-1 wild type or mutant K498R/K505R/K508R/K521R/K524R ((*mutKQR*) (2 μg) along with a luciferase reporter under the control of the endogenous MIP-2 (1 μg) (*C*) or iNOS (3 μg) (*D*) promoters; cells were subsequently treated for 4 h with TNFα (10 ng/ml) or LPS/IFNγ (0.05 μg/ml/100 units) in the simultaneous presence of low doses of deacetylase inhibitors (*HDi*; 10 nM TSA/200 μM NAM). Cells were harvested 24 h after transfection and NF-κB-dependent gene expression was determined as described in Fig. 1*C*.

subunits as indicated in Fig. 4*C*. After extensive washes, bound proteins were resolved by SDS-PAGE followed by autoradiography analysis for Mediator subunits. PARP-1 directly bound to CDK8 and MED14, although to a low extent, but not to the other tested subunits (Fig. 4*C*). We next tested whether PARP-1 might also directly interact with other components of the RNA polymerase II machinery. Recombinant purified HMG(YI), CDK8, cyclin C, TFIIF/RAP74, TFIIF/RAP30, TATA box-binding protein (TBP), and TBP-associated factors fused to GST were bound to glutathione beads followed by incubation with recombinant baculo-purified PARP-1 (Fig. 4*D*, *left* and *right panels*). After extensive washes, bound proteins were resolved by SDS-PAGE followed by immunoblot analysis for PARP-1. PARP-1 was able to bind directly to HMG(YI), CDK8, and TFIIF/RAP74 but not to the other factors tested (Fig. 4*D*, *left* and *right panels*).

Acetylation of PARP-1 at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 Stabilizes the Interaction of PARP-1 with p50—To investigate whether acetylation of PARP-1 mechanistically influences protein-protein interactions, we first tested which of the interaction partners associates with the acetylated domain of PARP-1. Different domains of PARP-1 fused to GST were bound to glutathione beads and incubated with purified p300, PARP-1, CDK8/cyclin C, p65, p50, TFIIF/RAP74 or *in vitro* transcribed/translated and radioactive-labeled MED14 (Fig. 5, *A* and *B*). After extensive washes, bound proteins were resolved by SDS-PAGE followed by immunoblot analysis with the indicated antibodies (Fig. 5*A*) or autoradiography (Fig. 5*B*). p300, PARP-1, CDK8, p65, p50, TFIIF/RAP74, and MED14 bound to different domains of PARP-1 as schematically drawn in Fig. 5*C*. PARP-1 interacted with a region between amino acids 1 and 214 as well as between amino acids 465 and

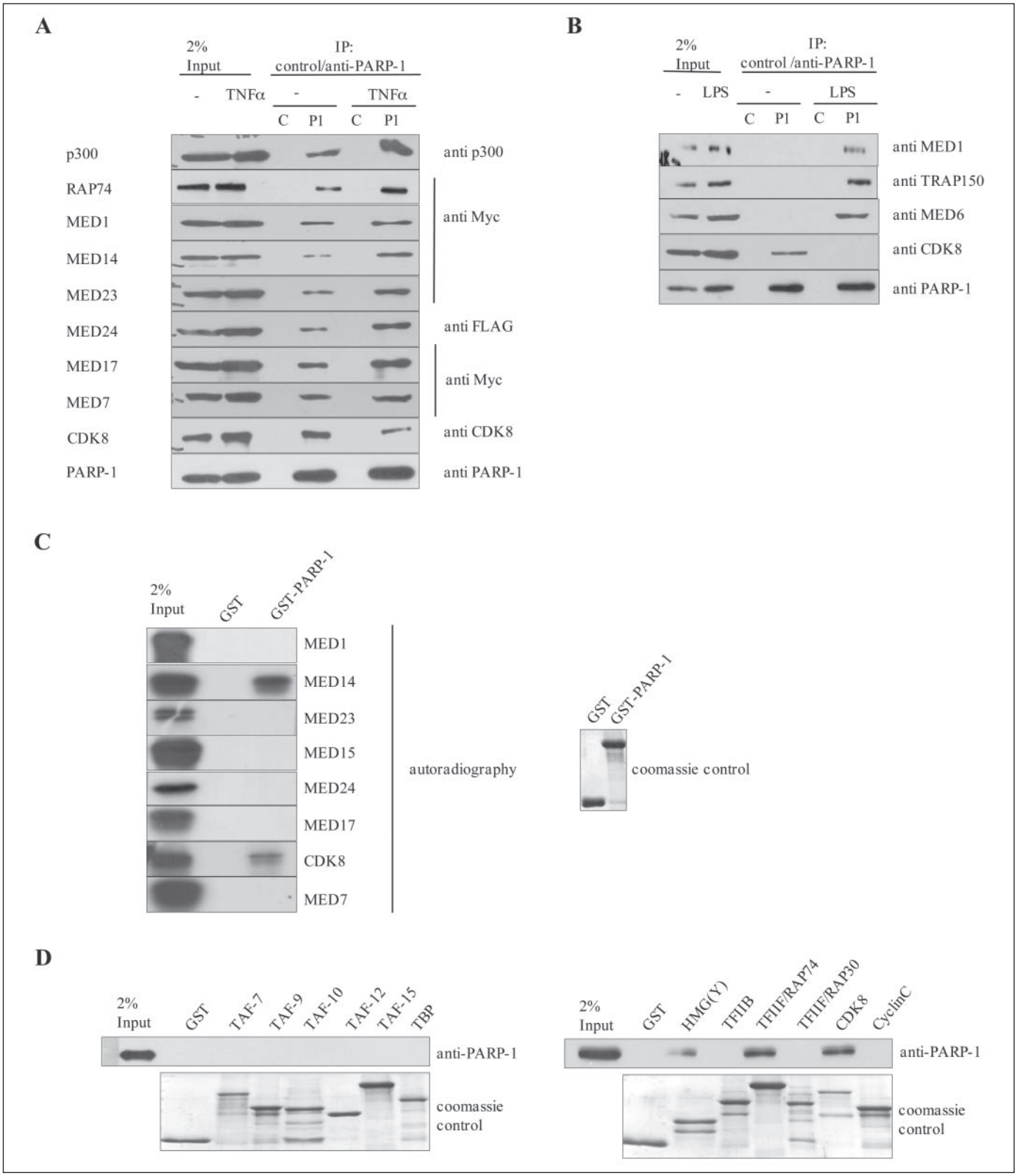


FIGURE 4. **PARP-1 interacts with the Mediator complex *in vivo* and directly with the Mediator subunits MED14 and CDK8 *in vitro*.** *A*, Myc- or FLAG-tagged Mediator subunits MED-1, MED-7, MED-14, MED-17, MED-23, and MED-24 were overexpressed in 293 cells and treated as indicated, and PARP-1 complexes were immunoprecipitated (*IP*) under physiological salt conditions from nuclear extracts using an anti-PARP-1 antibody and tested for PARP-1 and Myc- or FLAG-tagged Mediator subunits, p300, and CDK8 by immunoblot analysis using anti-PARP-1, anti-p300, anti-CDK8, anti-Myc, or anti-FLAG antibodies. *C*, control IgG; P1, anti-PARP-1. *B*, primary macrophages were treated as indicated with LPS (0.2 μg/ml) for 1 h. PARP-1 was immunoprecipitated under physiological salt conditions from nuclear extracts, and the presence of Mediator subunits was subsequently tested by immunoblot analysis using anti-MED1, anti-MED6 anti-TRAP150, anti-CDK8, or anti-PARP-1 antibodies. *C*, GST pull down assays under physiological salt conditions with PARP-1 full-length fused to GST (3 μg GST fusion protein) and the indicated *in vitro* transcribed/translated Mediator subunits. Bound proteins were resolved by SDS-PAGE followed by autoradiography. The Coomassie control gel is shown in the *left panel*. *D*, GST pull down assays under physiological salt conditions with the indicated general transcription factors and CDK8/cyclin C fused to GST (1–4 μg of GST fusion protein) and baculo-purified PARP-1 (0.5 μg input). Bound proteins were resolved by SDS-PAGE followed by immunoblot analysis using anti-PARP-1 antibodies (*upper panel*). The corresponding Coomassie control gel is shown in the *lower panel*. *TBP*, TATA box-binding protein.

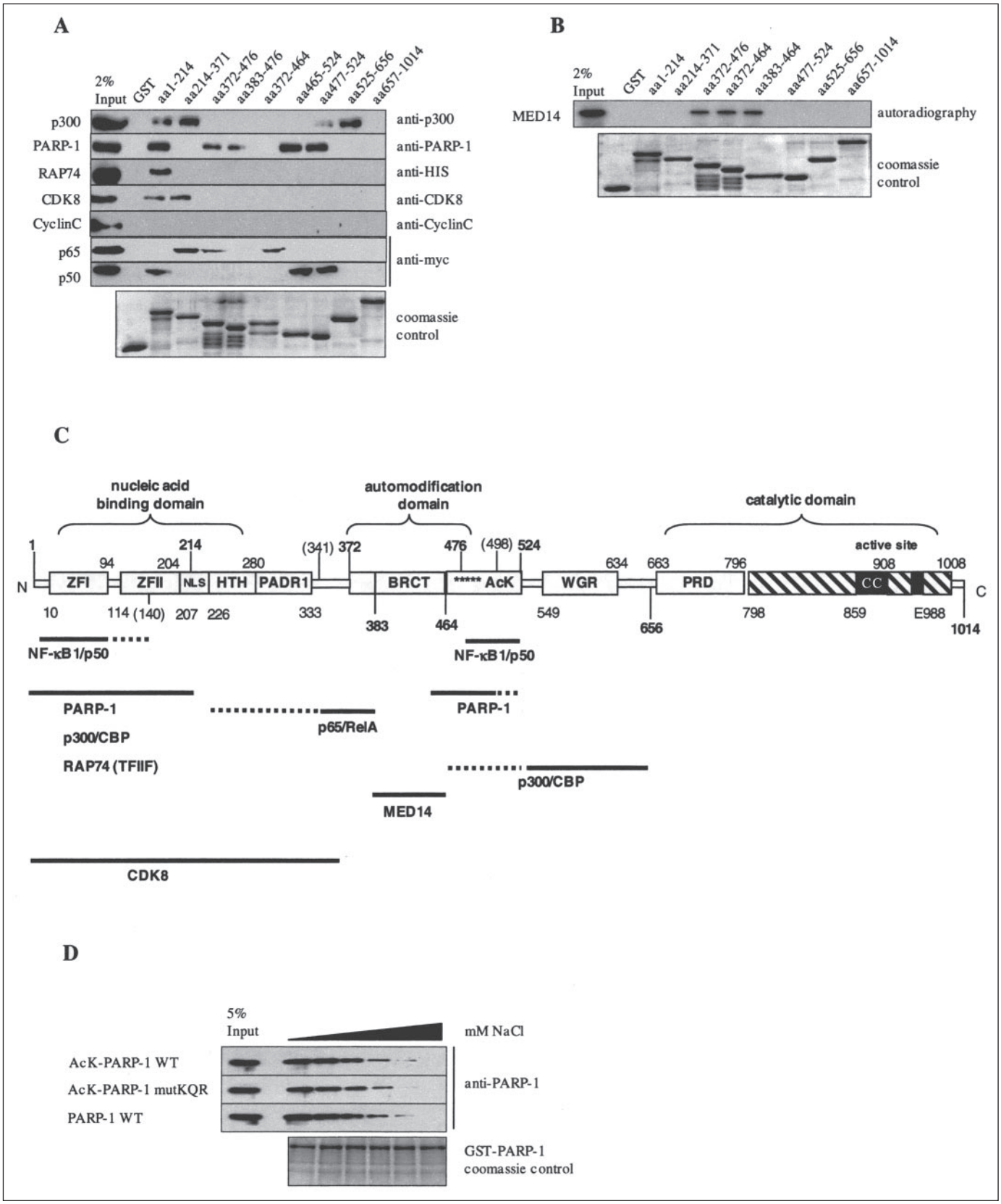


FIGURE 5. **Acetylation of PARP-1 stabilizes the interaction of PARP-1 with p50 but not p65 or PARP-1.** *A* and *B*, mapping of the interaction domains in PARP-1. Shown are GST pull down assays under high salt conditions with PARP-1 fragments fused to GST (1–3 μg of protein) as indicated and recombinant purified p300 (2 μg), PARP-1 (0.3 μg), RAP74 (1 μg), CDK8 (1 μg), cyclin C (0.5 μg), p65 (0.5 μg), and p50 (0.5 μg) (*A*) or *in vitro* transcribed/translated MED14 (*B*). Bound proteins were resolved by SDS-PAGE followed by immunoblot analysis with the corresponding antibodies (*A*) or autoradiography (*B*). The corresponding Coomassie control gel is shown in the *lower panels*. *aa*, amino acids. *C*, schematic picture of the PARP-1 interaction map: ZF I and ZFII, zinc finger 1 and 2; *NLS*, nuclear localization signal; *HTH*, helix turn helix domain, *BRCT*, C-terminal domain of a breast cancer susceptibility protein (BRCA); *WGR*, central WGR motif-containing domain;*PADR1*, PARP regulatory domain; *CC*, catalytic center. *D* and *E*, acetylation-dependent interaction of PARP-1 and p50. PARP-1 (*D*) or p50 (*E*) fused to GST (3 μg/2 μg) were incubated with *in vitro* acetylated or non-acetylated baculo-purified PARP-1 wild type (*WT*) or mutant form mutKQR (500 ng) in

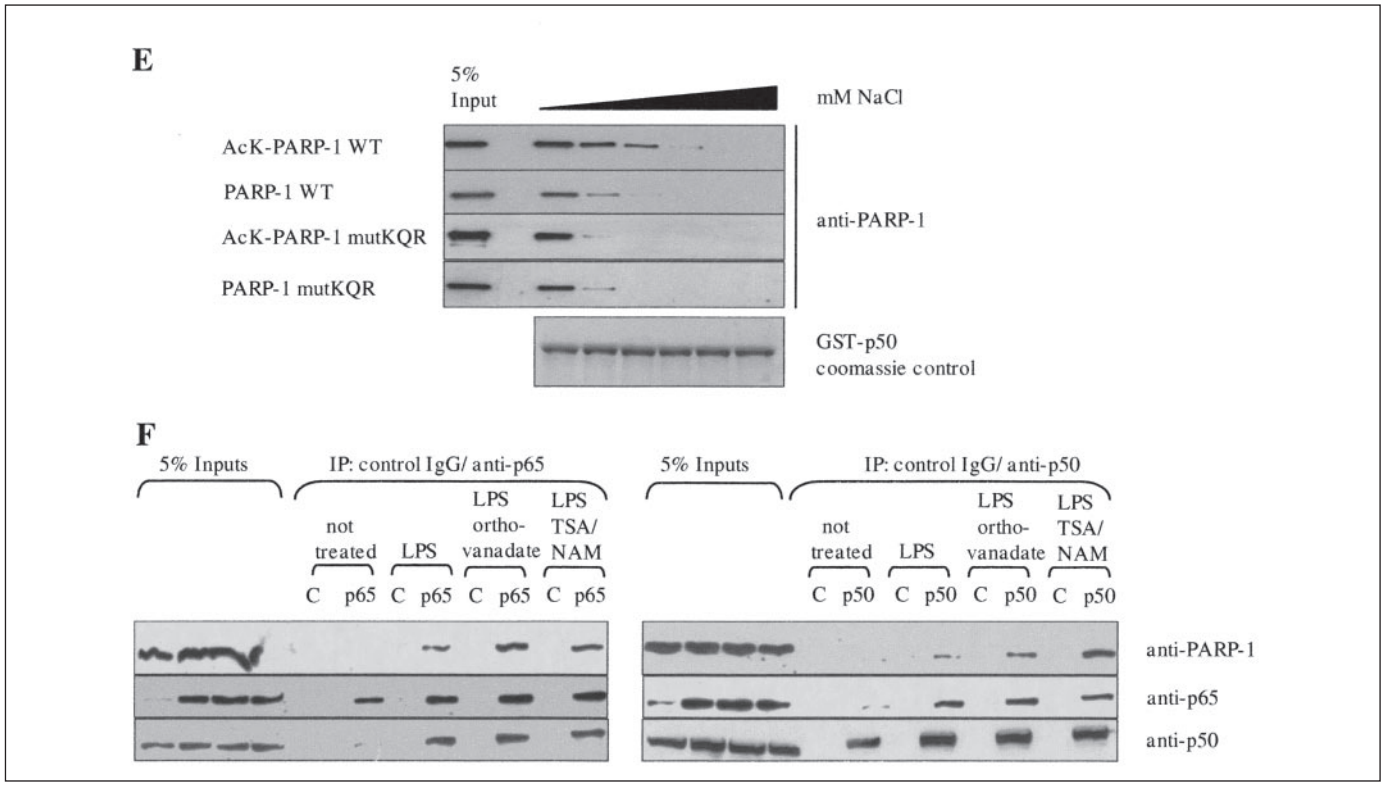


FIGURE 5—*continued*

524 (Fig. 5*C*). MED14 was the only tested protein that bound to the BRCT (carboxyl-terminal domain of a breast cancer susceptibility protein (BRCA)) domain of PARP-1. We have previously shown that p50 and p65 interact with a region between amino acids 341 and 531 of PARP-1 (20). In addition, p50 interacted also with a region between amino acids 1 and 140 containing the zinc finger I. Our more detailed interaction analysis suggests that p65 would interact with a region between amino acids 341 and 383 of PARP-1. p50 would interact with zinc finger I or zinc finger II and with a region between amino acids 477 and 524 (Fig. 5*C*). Together, these experiments revealed that only the interaction of PARP-1 with PARP-1 itself or p50 might be influenced by acetylation of PARP-1.

To test this hypothesis, recombinant purified PARP-1 or p50 fused to GST were bound to glutathione beads and incubated with non-acetylated or *in vitro* acetylated purified PARP-1 wild type and the mutant form mutKQR in the presence of increasing concentrations of NaCl as indicated (Fig. 5, *D* and *E*). After extensive washes, bound proteins were resolved by SDS-PAGE followed by immunoblot analysis for PARP-1 (Fig. 5, *D* and *E*). No significant differences between the acetylated or non-acetylated form of PARP-1 were observed for the dimerization or potential tetramerization of PARP-1 (Fig. 5*D*). Surprisingly, *in vitro* acetylated PARP-1 bound significantly better to p50 than did the non-acetylated PARP-1 (Fig. 5*E*). These results suggest that PARP-1 would dimerize or potentially tetramerize through the regions between amino acid 1 and 214 as well as between amino acid 465 and 497, whereas the region between amino acid 477 and 524 is required for acetylation-dependent interaction with p50 (Fig. 5*C*).

To confirm these *in vitro* data and to investigate whether acetylation would influence the stimuli-dependent complex formation between subunits of NF-κB and PARP-1 *in vivo*, we coimmunoprecipitated the NF-κB subunits p65 and p50 from nuclear extracts upon treatment of THP-1 with LPS in the presence or absence of deacetylase or phosphatase inhibitors (Fig. 5*F*, *left* and *right panels*) and tested the presence of PARP-1 by immunoblot analysis using anti-PARP-1 antibodies. Remarkably, PARP-1 bound significantly better to p65 when cells were simultaneously treated with LPS and phosphatase inhibitors (Fig. 5*F*, *left panel*), whereas p50 bound significantly better to PARP-1 upon simultaneous treatment with LPS and deacetylase inhibitors (Fig. 5*F*, *right panel*). No significant differences between deacetylase or phosphatase inhibitors were observed for the interaction between p50 and p65 (Fig. 5*F*, *left* and *right panels*). Taken together, these results suggest that phosphorylation might mainly enhance the interaction between p65 and PARP-1, whereas acetylation mainly enhances the interaction between p50 and PARP-1.

PARP-1 Interacts with Members of HDAC Class I and Is Potentially Deacetylated by HDACs 1–3 in Vivo—Because acetylation of proteins is known to be a reversible modification *in vivo*, we next tested whether PARP-1 might physically interact with HDACs *in vivo*. Because of the low quality of commercially available anti-HDAC antibodies, we decided to perform these experiments with overexpressed Myc- or FLAG-tagged HDACs. We coimmunoprecipitated PARP-1 complexes from nuclear extracts of untreated 293 cells overexpressing Myc-tagged versions of HDAC-1, HDAC-2, or HDAC-3 (Fig. 6*A*) and FLAG-tagged versions of HDAC-4, HDAC-5 or HDAC-6 (Fig. 6*B*) and tested the

presence of increasing concentrations of NaCl (125 mM NaCl, 25 mM potassium acetate to 225 mM NaCl, 25 mM potassium acetate) as indicated. Bound proteins were resolved by SDS-PAGE followed by immunoblot analysis using a anti-PARP-1 antibody. *F*, acetylation and stimuli-dependent complex formation of PARP-1 and subunits of NF-κB *in vivo*. NF-κB subunits p65 and p50 were coimmunoprecipitated (*IP*) from nuclear extracts upon treatment of THP-1 with LPS in the presence or absence of deacetylase or phosphatase inhibitors (*left* and *right panels*) and tested for the presence of p50, p65, and PARP-1 by immunoblot analysis using anti-p50, anti-p65, and anti-PARP-1 antibodies. *C*, control.

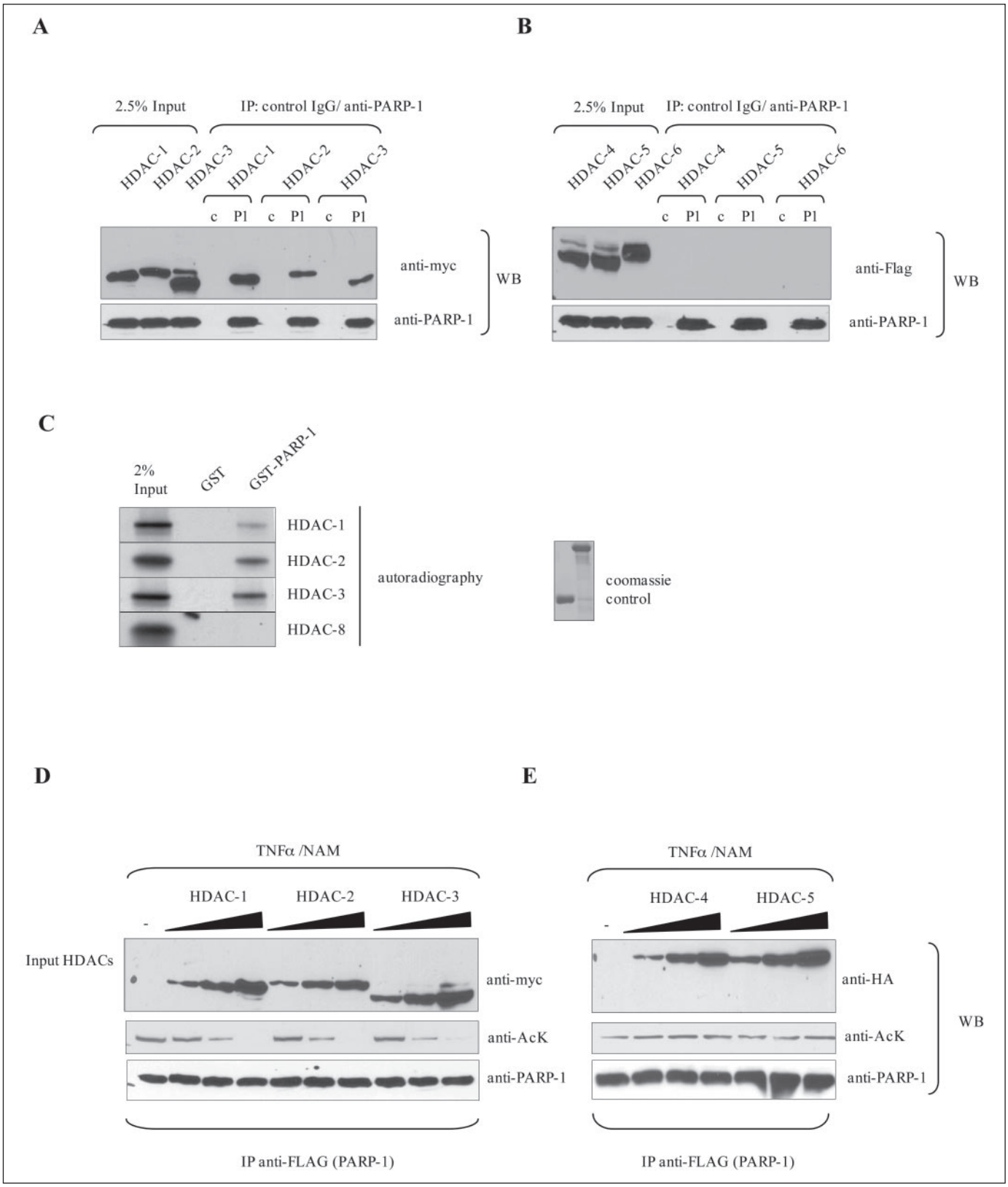


FIGURE 6. **PARP-1 interacts with members of HDAC class I and is potentially deacetylated by HDAC-1, HDAC-2, or HDAC-3 *in vivo*.** *A* and *B*, FLAG-tagged HDACs were overexpressed in 293 cells. PARP-1 complexes were co-immunoprecipitated (*IP*) from nuclear extracts of untreated 293 cells using an anti-PARP-1 (*P1*) antibody and subsequently tested for PARP-1 and Myc-tagged HDAC-1, HDAC-2, or HDAC-3 and FLAG-tagged HDAC-4, HDAC-5, or HDAC-6 by immunoblot (*WB*) analysis using anti-PARP-1, anti-Myc or anti-FLAG antibodies. *c*, control IgG. *C*, GST pull down assays under physiological salt conditions with PARP-1 fused to GST (4 μg of protein) and *in vitro* transcribed/translated HDAC-1, -2, -3, or -8. Bound proteins were resolved by SDS-PAGE followed by autoradiography (*left panel*). The corresponding Coomassie control gel is shown in the *right panel*. *D* and *E*, FLAG-tagged PARP-1 wild type was coexpressed with increasing amounts of Myc-tagged HDAC-1, HDAC-2, or HDAC-3 and hemagglutinin (*HA*)-tagged HDAC-4 or HDAC-5 in 293 cells. 24 h later cells were simultaneously treated with TNFα (10 ng/ml) and NAM (1 mM) for 30 min. FLAG-tagged PARP-1 was subsequently immunoprecipitated under high salt conditions from nuclear extracts of 293 cells, and the presence of acetylated forms of PARP-1 was tested by immunoblot analysis using anti-AcK or anti-FLAG antibodies.

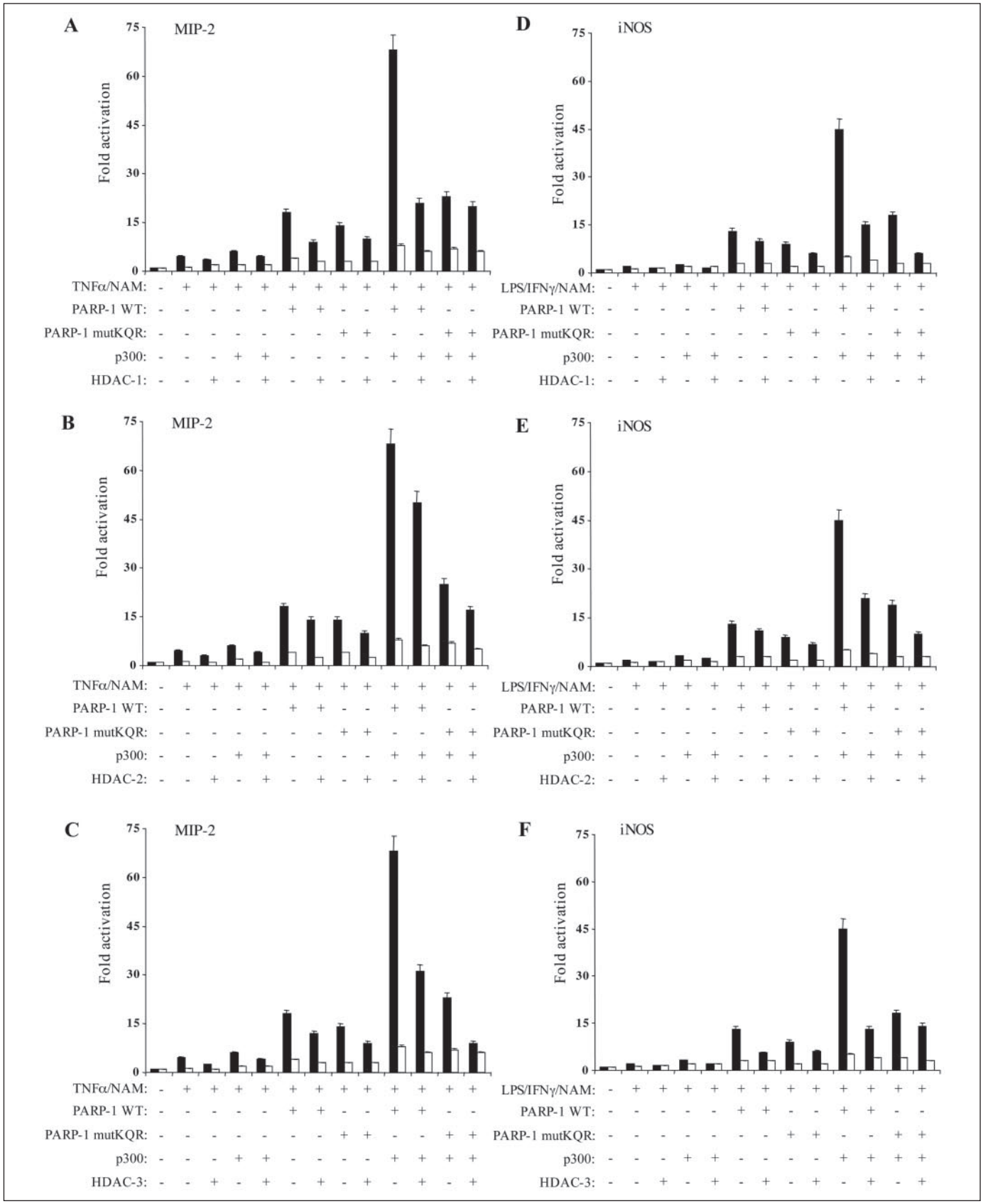


FIGURE 7. **The coactivator activity of PARP-1 is negatively regulated *in vivo* by HDAC-1 HDAC-2, or HDAC-3.** *A–F*, primary PARP-1(−/−) macrophages were cotransfected with expression vectors for RSV-nt-β-Gal (300 ng), PARP-1 wild type (*WT*) or mutant mutKQR (2 μg), p300 (2 μg), HDAC-1, HDAC-2, or HDAC-3 (2.5 μg each) along with a luciferase reporter under the control of the endogenous MIP-2 (1 μg) (*A–C*) or iNOS promoters (3 μg) (*D–F*); cells were subsequently treated for 4 h with TNFα (10 ng/ml) or LPS/IFNγ (0.05 μg/ml/100 units) in the simultaneous presence of low doses of deacetylase inhibitor (500 μM NAM). Cells were harvested 24 h after transfection, and NF-κB-dependent gene expression was determined as described in Fig. 1*C*.

presence of these HDACs by immunoblot analysis using anti-PARP-1, anti-Myc, or anti-FLAG antibodies. Interestingly, PARP-1 interacted with HDAC-1, HDAC-2, and HDAC-3 but not with HDAC-4, HDAC-5, or HDAC-6 (Fig. 6, *A* and *B*). DNA did not mediate the association of PARP-1 with HDACs since the presence of ethidium bromide or DNase I did not affect the interaction (data not shown). Because these results suggested that PARP-1 would directly interact with at least one of these HDACs, GST-PARP-1 full-length was bound to glutathione beads and incubated with *in vitro* translated and radioactive-labeled HDAC class I members HDAC-1, HDAC-2, HDAC-3, or HDAC-8 (Fig. 6*C*). After extensive washes, bound proteins were resolved by SDS-PAGE followed by autoradiography analysis for HDACs. PARP-1 bound directly to HDAC-1, HDAC-2, and HDAC-3 but not to HDAC-8 (Fig. 6*C*).

To investigate whether PARP-1 might be deacetylated by HDAC-1, HDAC-2, or HDAC-3 *in vivo*, FLAG-tagged PARP-1 wild type was coexpressed with increasing amounts of Myc-tagged HDAC-1, HDAC-2, or HDAC-3 (Fig. 6*D*) and hemagglutinin-tagged HDAC-4 or HDAC-5 in 293 cells (Fig. 6*E*). FLAG-tagged PARP-1 was immunoprecipitated from nuclear extracts under high salt conditions upon treatment of cells with TNFα and NAM. The presence of acetylated forms of PARP-1 was tested by immunoblot analysis using anti-AcK or anti-FLAG antibodies. These experiments revealed that PARP-1 might be deacetylated *in vivo* by HDAC-1, HDAC-2, or HDAC-3 (Fig. 6, *D* and *E*).

PARP-1-dependent Transcriptional Activation of NF-κB Seems to Be Negatively Regulated by HDACs 1–3 in Vivo—Next, we tested which HDAC has the strongest influence on the transcriptional coactivator activity of PARP-1 and might therefore also act functionally as a deacetylase for PARP-1 *in vivo*. PARP-1(−/−) cells were cotransfected with expression vectors for PARP-1 wild type or PARP-1 mutant form; that is, mutKQR, p300, HDAC-1, HDAC-2, or HDAC-3 along with a luciferase reporter under the control of the endogenous MIP-2 (Fig. 7, *A–C*) or iNOS promoters (Fig. 7, *D–F*). Cells were simultaneously treated with the indicated stimuli (TNFα or LPS/IFNγ) and low doses of deacetylase inhibitors (Fig. 7, *A–F*). Coexpression of p300 with HDAC-1, HDAC-2, or HDAC-3 in the absence of PARP-1 resulted in a reduced activation of NF-κB-dependent transcriptional activation (Fig. 7, *A–F*). No significant differences between HDAC-1, HDAC-2, or HDAC-3 were observed in the absence of PARP-1 for either MIP-2 or iNOS promoters (Fig. 7, *A–F*), indicating that the residual activation of NF-κB in PARP-1(−/−) cells in the absence of PARP-1 is equally repressed by HDAC-1, HDAC-2, or HDAC-3. Similar results were obtained in presence of PARP-1 wild type, although the repression by HDAC-2 was weaker when compared with HDAC-1 and HDAC-3 (Fig. 7, *A–F*). Remarkably, the repression by HDAC-1, HDAC-2, or HDAC-3 was strongly reduced when HDAC-1 HDAC-2, or HDAC-3 was coexpressed with PARP-1 mutant mutKQR (Fig. 7, *A–F*). Together these results suggest that HDAC-1, HDAC-2, and HDAC-3 might repress NF-κB-dependent transcription in part through deacetylation of PARP-1.

DISCUSSION

Growing experimental evidence suggests that PARP-1 can function as a promoter-specific coactivator (17). PARP-1 was also identified as an interaction partner not only of NF-κB but also of several sequence-specific transcription factors and cofactors including E2F1, Oct-1, and PC3/topoisomerase-I (17, 33) and has been shown to increase the transcriptional activity of these transcription factors (17). In 1997 Meisterernst *et al.* (34) identified human PARP-1 as one active component of the upstream stimulatory activity (USA)-derived positive cofactor complex PC-1 (34). The crude precursor human USA fraction consists of PC1/PARP-1, PC2/Mediator-like complex, PC3/topoisomerase-I, PC4/single-stranded DNA-binding protein, PC52, PC6, and HMG2 (34, 35). Earlier studies showed that full activation of transcription by NF-κB, Sp1, and Oct-1 in cell-free systems required a crude precursor USA coactivator fraction in addition to general transcription factors (36). Because PC1/PARP-1, PC3, and PC4 are all sequence-unspecific DNA-binding proteins, it was suggested that PC1/PARP-1 provide together with the other USA-derived positive cofactors PC3, PC4, PC52, and HMG2 a mainly structural/architectural role in assembling and stabilizing the pre-initiation complex by affecting the accessibility of RNA polymerase II to chromatin (17, 34, 35, 37).

The aim of this study was to investigate whether the coactivator activity of PARP-1 might be regulated by post-translational modifications such as acetylation. We provide both biochemical and functional evidence that acetylation of PARP-1 is required for its NF-κB coactivator activity.

PARP-1 Requires the Enzymatic Activity of p300 for Full NF-κB-dependent Transcriptional Activity—Several reports demonstrated that coactivator activity of PARP-1 for NF-κB-dependent gene expression seems to be dependent on the stimuli and cell type (17). We observed an impaired expression in primary PARP-1(−/−) macrophages of iNOS, KC, and MIP-2 genes but not of IκBα and IL-6 upon stimulation with TNFα or LPS/IFNγ, indicating that PARP-1 acts in a promoter-specific manner, similar to other coactivators of NF-κB such as CARM1 (16). PARP-1 and p300/CBP were shown to form a complex and function synergistically to enhance NF-κB-mediated gene expression (8). Moreover, we recently provided evidence that the enzymatic activities of p300/CBP and protein arginine methyltransferase CARM1 are required for NF-κB-dependent gene expression *in vivo* (16). NF-κB-dependent reporter gene analysis using the PARP-1-dependent MIP-2 and iNOS promoters revealed that the cooperativity between p300 and PARP-1 was severely impaired when an enzymatic mutant of p300 was coexpressed, indicating that the enzymatic activity of p300/CBP is required for NF-κB-dependent transactivation of PARP-1-dependent promoters upon treatment with inflammatory stimuli.

PARP-1 Is Acetylated in Vivo at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 by p300/CBP upon Stimulation—Growing experimental evidence has accumulated that NF-κB-dependent gene expression is also regulated by post-translational modifications such acetylation (22). This acetylation-dependent regulation was shown to occur at multiple levels (38). Acetylation of histones regulates the NF-κB-dependent gene accessibility (22). Moreover, direct acetylation of the NF-κB subunits p65 and p50 was shown to regulate transcriptional activation of NF-κB (39–41). Finally, other acetylation events were suggested to temporarily modulate the duration of NF-κB presence in the nucleus and DNA binding activity as well as protein-protein interactions with several cofactors involved in the transcriptional activity of NF-κB (22, 38). It was recently reported that the USA-derived positive cofactor PC4 is specifically acetylated by p300, thereby stimulating its double-stranded DNA binding activity which correlates with its coactivation activity (42, 43). Here we show that PARP-1 is acetylated *in vitro* and *in vivo* by p300 and CBP. Combined analysis by microcapillary reverse-phase high performance liquid chromatography nanoelectrospray tandem mass spectrometry and coexpression of p300/CBP and PARP-1 wild type or mutant revealed that PARP-1 is acetylated *in vivo* at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 by p300/CBP upon stimulation. *In vitro* acetylation experiments using purified recombinant PARP-1 wild type or mutant forms supported these *in vivo* observations.

Acetylation of PARP-1 at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 Is Required for NF-κB-dependent Transcriptional Activity in Vivo—The importance of PARP-1 acetylation for NF-κB-dependent transcription is supported by complementation experiments in primary PARP-

1(−/−) macrophages using the PARP-1 mutant mutKQR (K498R/K505R/K508R/K521R/K524R). Full synergistic enhancement of transcription regulated from the MIP-2 and iNOS promoters was obtained only when PARP-1 wild type was coexpressed with p300 in PARP-1(−/−) macrophages. The observed residual cooperative effects in the presence of mutated κB sites on the promoter most likely reflect the coactivator activity of PARP-1 on other sequence-specific transcription factors such as activator protein-1, Sp-1, Oct-1, interferon regulatory factors, or signal transducers and activators of transcription (STATs), which are also important for full activity of NF-κB on these promoters (24, 44–46). Several recent reports provided evidence that PARP-1 might also act as a coactivator of AP-1 and STATs (47–49). Interestingly, the observed differences between PARP-1 wild type and mutant mutKQR regarding the co-operativity of p300 and PARP-1 were less significant in experiments using reporter plasmids with mutated κB sites, suggesting that acetylation of PARP-1 might be mainly required for the NF-κB-dependent promoter activity. However, it remains to be further investigated whether acetylation of PARP-1 could also strongly influence other sequence-specific transcription factors or cofactors under certain conditions. Remarkably, coexpression of PARP-1 wild type or mutant mutKQR with p300 and Mediator subunits in PARP-1(−/−) macrophages demonstrated that acetylation of PARP-1 is also required for the transcriptional cooperativity between p300/CBP, Mediator, and PARP-1 on these promoters. Recent reports showed that ARC/Mediator interacts with the transactivation domain of p65 and enhances chromatin-dependent transcriptional activation by p65 *in vitro* (50). The Mediator complex is thought to provide the penultimate step in the activation process by bridging a given activator to RNA polymerase II and forming a scaffold onto which RNA polymerase II and general transcription factors can assemble and initiate transcription (51). Based on the multistep interaction model of transcriptional activation proposed by Roeder and co-workers (51), it was suggested that PC1/PARP-1 might facilitate together with other structural/architectural positive cofactors the co-operative interactions between sequence-specific activators and different coactivator complexes such as p300/CBP and Mediator, thereby providing an architectural function in stabilizing the pre-initiation complex (17). In our current studies we provided evidence that PARP-1 interacts *in vivo* with the Mediator complex under physiological conditions. Furthermore, PARP-1 directly interacted *in vitro* with the Mediator subunits MED14 and CDK8 as well as the TFIIF subunit RAP74 but not with TFIIB, TATA box-binding protein (TBP) and the tested TBP-associated factors. These results are consistent with the observation of Meisterernst *et al.* (34) that in "*in vitro* transcription" assays, PARP-1 is only required during assembly of RNA polymerase II and general transcription factors on preformed TFIID-TFIIA-DNA complexes (34). In these studies PARP-1 was unable to stimulate the formation of a TFIID-promoter complex. In addition, PARP-1 stimulated transcription only when added before the complete pre-initiation complex was formed, which implies that PARP-1 might only function during assembly of the pre-initiation complex (34).

Whether acetylation of PARP-1 might regulate the PARP-1 activity at this level in the context of chromatin remains to be investigated. However, it seems unlikely since the Mediator subunits DRIP150 and CDK8 did not bind to the acetylated domain in PARP-1. Surprisingly, acetylation of PARP-1 regulates the stimuli-dependent interaction of PARP-1 with NF-κB1 subunit p50 but not p65. Because the acetylation mutant mutKQR of PARP-1 could still partially coactivate NF-κB, acetylation of PARP-1 might be mechanistically required for the stabilization of preformed PARP-1 containing transcriptional coactivator-cofactor complexes.

PARP-1-dependent Transcriptional Activation of NF-κB Seems to Be Negatively Regulated in Vivo by HDACs 1–3—Overexpression of HDAC-1, HDAC-2, and HDAC-3 was shown to repress NF-κB-dependent transcription upon treatment with inflammatory stimuli (22, 38, 41). HDAC-3 was shown to be required for the deacetylation of p65 (52). HDAC-1, HDAC-2, and HDAC-3 interact directly with several proteins involved in the NF-κB signaling pathway, including NF-κB itself (22, 52, 53). Interestingly, PARP-1 formed a complex with HDAC-1, HDAC-2, or HDAC-3 in the nucleus but not with HDAC-4, HDAC-5, or HDAC-6. The direct interaction *in vitro* between PARP-1 and HDACs 1–3 was weak and might be very transient or regulated *in vivo* by other unidentified post-translational modifications. Subsequent *in vivo* deacetylation experiments with increasing amounts of overexpressed HDACs suggested that deacetylation of PARP-1 *in vivo* might be mediated by HDAC-1, HDAC-2, or HDAC-3. Transient reporter assays revealed that the PARP-1-dependent transcriptional activity of NF-κB is negatively regulated *in vivo* by HDAC-1, HDAC-2, or HDAC-3. Because transiently transfected plasmids seem not to be properly chromatinized, nucleosomal histones are unlikely to represent the only relevant substrates whose lysine acetylation is required for NF-κB-dependent transcription. Remarkably, when HDAC-1, HDAC-2, or HDAC-3 was co-expressed with PARP-1 mutant mutKQR, no significant additional promoter-specific decrease in NF-κB-dependent transcription was obtained. These results suggest that HDAC-1, HDAC-2, and HDAC-3 might deacetylate promoter-bound PARP-1 or other unidentified cofactors or coactivators dependent on acetylation of PARP-1.

Interestingly, several recent reports demonstrated that inhibition of HDACs could selectively suppress transcription in a gene-specific manner by inducing an elongational arrest and/or premature termination between exons 1 and 2 (54–57). Thus, in the context of chromatin, it might be that for at least a subset of NF-κB-dependent genes, deacetylation of NF-κB itself or of coactivators involved in the nuclear activity of NF-κB, such as PARP-1, could be required for transcriptional elongation or re-initiation. For at least a subset of PARP-1-dependent NF-κB target genes, both the enzymatic activities of p300 and acetylation of PARP-1 are required for transcriptional activity. However, the exact molecular mechanism by which acetylation of PARP-1 regulates the coactivator activity of PARP-1 in the context of chromatin remains to be investigated. In addition, we cannot exclude the possibility that other histone acetyltransferases might acetylate PARP-1 on other lysine residues and thereby influence its activities. Whether p65 or other sequence-specific transcription factors known to cooperate with NF-κB in gene induction could regulate the acetylation of PARP-1 is currently under investigation.

Taken together, these results support the hypothesis that the different physiological functions of PARP-1 are regulated by post-translational modifications such as acetylation in a stimuli-specific manner.

Acknowledgments—We are grateful to Dr. Z. Q. Wang (International Agency for Research on Cancer, Lyon, France) for the 129S/EV-PARP-1(+/+) and 129S/EV-PARP-1(−/−) mice breeding pairs, Drs. W. L. Kraus (Cornell University, New York), D. Thanos (Institute of Molecular Biology and Genetics, Alexander Fleming Biomedical Sciences Research Center, Athens, Greece), S. L. Schreiber (Harvard University, Cambridge, MA), H.-J. Kwon, (Yonsei University, Seoul, Korea), M. A. Perrella (Harvard School of Public Health, Boston, MA), R. M. Evans (The Salk Institute for Biological Studies, La Jolla, CA), Dr. M. Otsuka (University of Tokyo, Japan), Dr. Z. F. Burton (Michigan State University, East Lansing, MI), Dr. G. Napolitano (University of Naples "Frederico II", Naples, Italy), Dr. R. Pinhero (University of Guelph, Ontario, Canada), and Dr. L. Freedman (Merck Research Laboratories, West Point, NY) for the generous provision of reagents. We also thank the members of the Institute of Veterinary Biochemistry and Molecular Biology (University of Zurich, Switzerland) for helpful advice and comments.

REFERENCES

1. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16,** 225–260
2. Karin, M. (1998) *Cancer J. Sci. Am.* **4,** Suppl. 1, 92–99
3. Ghosh, S., and Karin, M. (2002) *Cell* **109,** (suppl.) 81–96
4. Karin, M., Yamamoto, Y., and Wang, Q. M. (2004) *Nat. Rev. Drug Discov.* **3,** 17–26
5. Karin, M., and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* **18,** 621–663
6. Merika, M., Williams, A. J., Chen, G., Collins, T., and Thanos, D. (1998) *Mol. Cell* **1,** 277–287
7. Perkins, N. D. (1997) *Int. J. Biochem. Cell Biol.* **29,** 1433–1448
8. Hassa, P. O., Buerki, C., Lombardi, C., Imhof, R., and Hottiger, M. O. (2003) *J. Biol. Chem.* **278,** 45145–45153
9. Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) *Science* **275,** 523–527
10. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell* **1,** 661–671
11. Goodman, R. H., and Smolik, S. (2000) *Genes Dev.* **14,** 1553–1577
12. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87,** 953–959
13. Schiltz, R. L., Mizzen, C. A., Vassilev, A., Cook, R. G., Allis, C. D., and Nakatani, Y. (1999) *J. Biol. Chem.* **274,** 1189–1192
14. Kundu, T. K., Palhan, V. B., Wang, Z., An, W., Cole, P. A., and Roeder, R. G. (2000) *Mol. Cell* **6,** 551–561
15. Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., and Collins, T. (1999) *Mol. Cell. Biol.* **19,** 6367–6378
16. Covic, M., Hassa, P. O., Saccani, S., Buerki, C., Meier, N. I., Lombardi, C., Imhof, R., Bedford, M. T., Natoli, G., and Hottiger, M. O. (2005) *EMBO J.* **24,** 85–96
17. Hassa, P. O., and Hottiger, M. O. (2002) *Cell. Mol. Life Sci.* **59,** 1534–1553
18. Hassa, P. O., and Hottiger, M. O. (1999) *Biol. Chem.* **380,** 953–959
19. Petrilli, V., Herceg, Z., Hassa, P. O., Patel, N. S., Di Paola, R., Cortes, U., Dugo, L., Filipe, H. M., Thiemermann, C., Hottiger, M. O., Cuzzocrea, S., and Wang, Z. Q. (2004) *J. Clin. Investig.* **114,** 1072–1081
20. Hassa, P. O., Covic, M., Hasan, S., Imhof, R., and Hottiger, M. O. (2001) *J. Biol. Chem.* **276,** 45588–45597
21. Tulin, A., and Spradling, A. (2003) *Science* **299,** 560–562
22. Chen, L. F., and Greene, W. C. (2004) *Nat. Rev. Mol. Cell Biol.* **5,** 392–401
23. Perrella, M. A., Pellacani, A., Wiesel, P., Chin, M. T., Foster, L. C., Ibanez, M., Hsieh, C. M., Reeves, R., Yet, S. F., and Lee, M. E. (1999) *J. Biol. Chem.* **274,** 9045–9052
24. Kim, D. S., Han, J. H., and Kwon, H. J. (2003) *Mol. Immunol.* **40,** 633–643
25. Pinhero, R., Liaw, P., and Yankulov, K. (2004) *Biol. Proced. Online* **6,** 163–172
26. Wang, Z. Q., Stingl, L., Morrison, C., Jantsch, M., Los, M., Schulze-Osthoff, K., and Wagner, E. F. (1997) *Genes Dev.* **11,** 2347–2358
27. Hottiger, M. O., Felzien, L. K., and Nabel, G. J. (1998) *EMBO J.* **17,** 3124–3134
28. Oh, Y. K., Park, J. S., Kang, M. J., Ko, J. J., Kim, J. M., and Kim, C. K. (2003) *Vaccine* **21,** 2837–2843
29. Ringenbach, L., Bohbot, A., Tiberghien, P., Oberling, F., and Feugeas, O. (1998) *Gene Ther.* **5,** 1508–1516
30. Tinsley, R. B., Vesey, M. J., Barati, S., Rush, R. A., and Ferguson, I. A. (2004) *J. Gene Med.* **6,** 1023–1032
31. Sterner, D. E., and Berger, S. L. (2000) *Microbiol. Mol. Biol. Rev.* **64,** 435–459
32. Pavri, R., Lewis, B., Kim, T. K., Dilworth, F. J., Erdjument-Bromage, H., Tempst, P., de Murcia, G., Evans, R., Chambon, P., and Reinberg, D. (2005) *Mol. Cell* **18,** 83–96
33. Simbulan-Rosenthal, C. M., Rosenthal, D. S., Luo, R., Samara, R., Espinoza, L. A., Hassa, P. O., Hottiger, M. O., and Smulson, M. E. (2003) *Oncogene* **22,** 8460–8471
34. Meisterernst, M., Stelzer, G., and Roeder, R. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2261–2265
35. Roeder, R. G. (1998) *Cold Spring Harbor Symp. Quant. Biol.* **63,** 201–218
36. Guermah, M., Malik, S., and Roeder, R. G. (1998) *Mol. Cell. Biol.* **18,** 3234–3244
37. Ge, H., Si, Y., and Roeder, R. G. (1998) *EMBO J.* **17,** 6723–6729
38. Schmitz, M. L., Mattioli, I., Buss, H., and Kracht, M. (2004) *Chembiochem* **5,** 1348–1358
39. Chen, L. F., and Greene, W. C. (2003) *J. Mol. Med.* **81,** 549–557
40. Deng, W. G., Zhu, Y., and Wu, K. K. (2003) *J. Biol. Chem.* **278,** 4770–4777
41. Chen, L. F., Mu, Y., and Greene, W. C. (2002) *EMBO J.* **21,** 6539–6548
42. Kumar, B. R., Swaminathan, V., Banerjee, S., and Kundu, T. K. (2001) *J. Biol. Chem.* **276,** 16804–16809
43. Kaiser, K., Stelzer, G., and Meisterernst, M. (1995) *EMBO J.* **14,** 3520–3527
44. Kim, Y. M., Ko, C. B., Park, Y. P., Kim, Y. J., and Paik, S. G. (1999) *Mol. Cell* **9,** 99–109
45. Ganster, R. W., Taylor, B. S., Shao, L., and Geller, D. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8638–8643
46. Diaz-Guerra, M. J., Velasco, M., Martin-Sanz, P., and Bosca, L. (1996) *J. Biol. Chem.* **271,** 30114–30120
47. Zingarelli, B., Hake, P. W., Burroughs, T. J., Piraino, G., O'Connor, M., and Denenberg, A. (2004) *Immunology* **113,** 509–517
48. Kiefmann, R., Heckel, K., Doerger, M., Schenkat, S., Kupatt, C., Stoeckelhuber, M., Wesierska-Gadek, J., and Goetz, A. E. (2004) *Intensive Care Med.* **30,** 1421–1431
49. Ha, H. C., Juluri, K., Zhou, Y., Leung, S., Hermankova, M., and Snyder, S. H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3364–3368
50. Naar, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W., and Tjian, R. (1999) *Nature* **398,** 828–832
51. Malik, S., Gu, W., Wu, W., Qin, J., and Roeder, R. G. (2000) *Mol. Cell* **5,** 753–760
52. Chen, L., Fischle, W., Verdin, E., and Greene, W. C. (2001) *Science* **293,** 1653–1657
53. Ashburner, B. P., Westerheide, S. D., and Baldwin, A. S., Jr. (2001) *Mol. Cell Biol.* **21,** 7065–7077
54. Hu, J., and Colburn, N. H. (2005) *Mol. Cancer Res.* **3,** 100–109
55. Blanchard, F., and Chipoy, C. (2005) *Drug Discov. Today* **10,** 197–204
56. Wilson, A. J., Velcich, A., Arango, D., Kurland, A. R., Shenoy, S. M., Pezo, R. C., Levsky, J. M., Singer, R. H., and Augenlicht, L. H. (2002) *Cancer Res.* **62,** 6006–6010
57. Tong, X., Yin, L., Joshi, S., Rosenberg, D. W., and Giardina, C. (2005) *J. Biol. Chem.* **280,** 15503–15509

10. Acknowledgements

First of all, I wish to thank Prof. Dr. Dr. Michael O. Hottiger for giving me the opportunity to perform the thesis in his lab, for his excellent guidance during my DVM work and refereeing my thesis.

I thank Prof. Dr. Felix R. Althaus for his interest in ongoing research and for refereeing my thesis.

Special thanks go to Dr. Paul O. Hassa for his excellent advice, for proofreading my thesis, for the fun we had and especially for his daily jokes.

Last but not least I would like to thank all my other colleagues in the Instiute of Veterinary Biochemistry and Molecular biology, past and present, for their help and for providing such a pleasant atmosphere.

Finally, I thank my family and all friends outside the lab for their moral support and interest in my work.

Curriculum vitae

Name:	Meier
First name:	Nadja Iveta
Date of birth:	22.8.1980
Place of birth:	Zurich, ZH, Switzerland
Citizenship:	Zurich, ZH, and Buchberg, SH, Switzerland

Education:

1987-1993	Primary school in Zurich-Seebach
1993-2000	Kantonsschule Oerlikon, Zurich
2000	Matura Typus B
2000-2006	Studies in veterinary medicine at the University of Zurich, Vetsuisse Faculty, Switzerland
2003-2006	Thesis in the group of Prof. Dr. M.O. Hottiger at the Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich. Supervised by Prof. Dr. M.O. Hottiger, Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich
2006	State examination at the University of Zurich, Vetsuisse Faculty, Switzerland